UNIVERSITY OF ALBERTA

INFLUENCE OF CELL ADHESIONS ON THE FREEZING RESPONSE OF BIOLOGICAL SYSTEMS

by

JASON PAUL ACKER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Pathology

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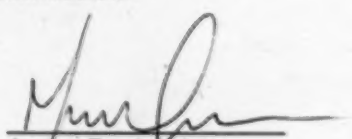

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This thesis is dedicated to the two greatest teachers in my life -- my parents.

ABSTRACT

While most cells in suspension can recover virtually full viability and function after freezing, this has not been the case for tissues. The complex, coordinated interactions between the multiple cells and cell types in tissues are potential sites of damage during freezing. Cryomicroscopy and video image analysis were used to examine the role of cell adhesions on the freezing response of cells in a tissue model system. A technique was developed using a fluorescent nuclear stain (SYTO) to quantitatively determine IIF in cell monolayers. Cell-cell and cell-surface interactions mediate the susceptibility of cells to intracellular ice formation (IIF). Using a dual fluorescent staining technique it was found that IIF did not result in the immediate disruption of the plasma membrane in the majority of cells in a monolayer. Induction of ice between adjacent cells via surface-catalyzed nucleation and gap junctions was responsible for the enhanced sensitivity of tissues to IIF. These observations are of theoretical interest in the study of the mechanisms of IIF and of practical interest in the cryopreservation and cryodestruction of tissues.

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TABLE OF CONTENTS

CHAPTER			PAGE
1.	**INTRODUCTION**		
	1.1	General Introduction	1
	1.2	Cellular Cryobiology	2
	1.3	Intracellular Ice Formation	5
	1.4	Traditional Cryopreservation of Cells	11
	1.5	Tissues	13
	1.6	Review of Cryopreservation of Tissues and Organs	14
	1.7	Hypothesis	18
	1.8	Objectives and Approach	18
	1.9	References	21
2.	**ROLE OF CELL CONTACT ON MEMBRANE INTEGRITY AFTER INTRACELLULAR ICE FORMATION**		
	2.1	Introduction	29
	2.2	Materials and Methods	32
		Cell Culture	32
		Cryomicroscope and Video System	33
		Membrane Integrity Assay	34
		Detection of Intracellular Ice	35
		Experimental Procedure	35
	2.3	Results	36
		Intracellular Ice Formation at Constant Temperature	36
		Detection of Intracellular Ice Formation	37
		Membrane Integrity After Thawing	38
	2.4	Discussion	38
	2.5	References	42

3. **THE ROLE OF CELL-CELL CONTACT ON INTRACELLULAR ICE FORMATION**

3.1	Introduction	55
3.2	Materials and Methods	57
	Cell Culture	57
	Freezing at Constant Subzero Temperature	57
	Image Analysis	58
	Statistical Analysis	58
3.3	Results	59
	Comparison of IIF Behavior in Cell Suspensions and Confluent Monolayers	59
	Influence of Cell-cell Contact on the Statistical Test	59
3.4	Discussion	60
3.5	References	62

4. **INTRACELLULAR NUCLEATION THROUGH GAP JUNCTIONS**

4.1	Introduction	71
4.2	Materials and Methods	74
	Cell Culture	74
	Detection of Intracellular Ice Formation	75
	Freezing at Constant Subzero Temperatures	75
4.3	Results	76
	Intracellular Ice Formation at Constant Temperature	76
	Ice Propagation in Monolayers	77
4.4	Discussion	77
4.5	References	81

5. **GENERAL DISCUSSION AND CONCLUSION**

5.1	Review of Thesis Objectives	87
5.2	Summary of Results	87
5.3	Significance to Cryobiology	89

LIST OF TABLES

		PAGE
Table 4.1	Rate of Ice Growth and Rate of Intercellular Nucleation in MDCK Monolayers as a Function of Temperature	86

LIST OF FIGURES

		PAGE
Figure 2.1	Schematic of a Cryomicroscope	48
Figure 2.2	Experimental Thermal Protocol	49
Figure 2.3	Cumulative Incidence of IIF as a function of Temperature - V-79W Hamster Fibroblasts	50
Figure 2.4	Photos of the Effects of IIF on pattern of SYTO stain	51
Figure 2.5a	Cumulative Incidence of IIF and EB Positive - V-79W cell suspensions	52
Figure 2.5b	Cumulative Incidence of IIF and EB Positive - V-79W single attached cells	53
Figure 2.5c	Cumulative Incidence of IIF and EB Positive - V-79W confluent monolayers	54
Figure 3.1	Statistical Analysis Summary	66
Figure 3.2	Cumulative Incidence of IIF in V-79W	67
Figure 3.3	Cumulative Incidence of IIF in V-79W as a Function of Time	68
Figure 3.4	Normalized Number of Connected Components as a Function of Temperature	69
Figure 3.5	Normalized Number of Connected Components as a Function of Time	70
Figure 4.1	Cumulative Incidence of IIF in V-79W and MDCK	85

LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

CCD	charge-coupled device
EB	ethidium bromide
FDA	flourescein diacetate
IIF	intracellular ice formation
MDCK	madin darby canine kidney
Me_2SO	dimethyl sulfoxide
MEM	minimum essential media
PBS	phosphate buffered saline
TCM	tissue culture media
uv	ultraviolet

SYMBOLS

$^{50}T_{IIF}$	temperature for 50% intracellular ice formation

CHAPTER 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

Spring in the Canadian prairies is a joyous and wonderful occasion. The days are longer, the weather is warmer, and a glimmer of new life peaks through its covering of snow and ice. After the long winter confined indoors, we cherish the opportunity to escape outside and appreciate this astounding transformation. To many, the dawn of spring signifies the approaching summer and long days spent on the patio or at the beach. However, to the more thoughtful individual, spring signals the beginning of one of nature's most amazing events. Trees, plants and animals emerge from their winter dormancy to resume where they left off the previous fall. The harsh temperatures and winter conditions was unable to prevent the rejuvenation of life in these organisms. Unfortunately, Canadians know too well the damaging effects of low temperatures. How then does nature protect itself from the bitter cold for such a lengthy period of time?

The universal component necessary for life to exist is water. Water is the most essential element in the maintenance of cellular structure and function. Solidification of water by freezing is disruptive and usually lethal. Yet paradoxically, the ability of biological material to survive freezing is a widespread natural phenomenon. Cryobiology is the study of the effects of low temperatures on biological systems. This branch of science, while seemingly limited in focus, incorporates a wide variety of disciplines. Physicists, biologists, chemists, engineers and physicians have all worked together to explore the effects of low temperatures on biological materials. It is from this cooperation that the enormous progress in cryobiology has lent its knowledge to a vast range of

subjects including: food studies, space sciences, cell physiology, electron microscopy, molecular biology and cell preservation.

Over the last 50 years, techniques have been developed which allow for the long-term preservation of cells. As a result of a more complete understanding of the intricate elements involved in the freezing of cells, it has been possible to predict with a relatively high degree of certainty, their response to freezing. This careful elucidation of the mechanisms by which cells are damaged has allowed for the routine cryopreservation of a variety of cell types.

Advances in cryobiology have facilitated the development of techniques for the preservation and storage of cellular and tissue systems at low temperatures. With an ever increasing clinical demand for transplantable tissues, cryopreservation is becoming more important in long term banking. Lengthy storage of cells and tissue allows for more effective donor-recipient matching, tissue typing and infectious disease testing, and national and international tissue sharing. However, of those tissues now routinely cryopreserved and transplanted, it is becoming increasingly apparent that the quality of the cryopreserved tissues needs to be improved. With post-thaw viability of these tissues being one of the major limiting factors in successful transplantation, a better understanding of the mechanisms of injury in these systems needs to be developed.

1.2 CELLULAR CRYOBIOLOGY

The formation of ice in the external environment of living cells induces numerous physical and chemical changes which they must be able to respond to in order to remain viable. Cell injury is related to the nature and kinetics of their response to these temperature-induced conditions.

Under normal physiological conditions, when a cell suspension is cooled below the freezing point of its solution, ice will form first in the extracellular space. As the cell membrane serves as an effective inhibitor to ice growth (1), and the cytoplasm contains few effective nucleators (2.3), ice is not immediately formed intracellularly. Water is removed in the form of ice resulting in the concentration of the extracellular solutes in the unfrozen fraction. The development of an osmotic pressure gradient across the cell membrane occurs, with the exterior having a higher osmotic pressure than the interior. This gradient provides the driving force for the efflux of water from the cells. Upon additional cooling, more ice will form extracellularly, and the cell will become increasingly dehydrated. If the cooling rate is sufficiently slow, the movement of water across the membrane will maintain the cell close to osmotic equilibrium with the external environment. In contrast, when cells are cooled rapidly, the formation of ice in the external solution is much faster than the efflux of water from the cell. This results in the cytoplasm becoming increasingly supercooled and the eventual formation of intracellular ice. The ability of a cell to react osmotically to the changing environment is therefore a fundamental element in its response to low temperatures.

A quantitative approach to describing a cells response to low temperatures was devised by Mazur in 1963 (4). He proposed that the rate of efflux of water from a cell during freezing could be predicted if one knows the permeability of the cell to water and its surface area to volume ratio. The expressions he developed, allowed for the determination of the degree of supercooling in a cell as a function of cooling rate. This information provided the means by which one could predict

the occurrence of damaging events, such as intracellular ice formation, and allowed for the design of methods which could avoid this injury.

Mazur's model of low temperature responses of cells allowed for a more detailed examination of the mechanisms by which living systems are injured during freezing. In 1972, Mazur, Leibo and Chu presented a two-factor hypothesis to explain the observed differences in cryoinjury during slow and rapid cooling (5). During slow cooling, they attributed cellular injury to an alteration in the properties of the intracellular and extracellular solution as a result of ice formation. Damage as a result of rapid cooling was proposed to occur as a result of the formation and recrystallization of intracellular ice. The development of this dual factor hypothesis for freezing injury served as the base from which numerous mechanistic studies of a cell's response to either slow or rapid cooling rates have branched.

During slow cooling, the cell is able to maintain osmotic equilibrium with the extracellular space through dehydration. While osmotic equilibrium is maintained and intracellular ice is avoided, damage can still occur. There have been two mechanisms by which damage is thought to occur during slow cooling: solute toxicity (5.6), and shrinkage damage (7.8). During slow cooling, the concentration of the intracellular and extracellular solutes occurs as a result of the efflux of water from the cells and the formation of external ice, respectively (4). While the molecular mechanism by which concentrated solutes damage a cell are relatively unknown, it is foreseeable that alterations in the chemical balance of the cell could result in numerous biophysical and biochemical alterations which could lead to cell death. Work in this area of cryobiology is still in its early stages of development. The second mechanism by which slow

cooling is thought to injure cells is by the shrinkage of the cell that results from exposure to hypertonic solutions. It is thought that the cell possess a minimum critical volume which cannot be violated during freezing (7). Alternatively, it has also been proposed that the surface area of the cell is reduced as a result of a loss in membrane material during slow cooling (8). Upon thawing, the cell expands to its previous isotonic volume, but as a result of exceeding the cells minimum critical volume, or the reduction in the cells surface area, injury occurs. Slow cooling injury is therefore a result of the toxic and osmotic events associated with exposure an increasing concentration of solutes.

Of interest to this thesis is the phenomenon of intracellular ice formation.

1.3 INTRACELLULAR ICE FORMATION

Intracellular ice formation occurs when a cell is unable to maintain equilibrium with the external environment. During rapid cooling, the formation of ice and the concentration of extracellular solutes occurs too quickly for the cell to respond by exosmosis. This results in the cytoplasm becoming increasingly supercooled and an increase in the probability of intracellular freezing. Supercooling however, is not the only prerequisite condition for IIF. The supercooled cytoplasm must be nucleated or seeded if ice is to form. As the cell membrane serves as an effective inhibitor to ice growth (1), and the cytoplasm contains few effective nucleators (2,3), the mechanism by which the supercooled cytoplasm becomes nucleated has been the subject of much debate.

There is strong evidence to suggest that extracellular ice and the cell plasma membrane are involved in the initiation of IIF. Most cells in an isotonic solution will freeze intracellularly between -5 and -15°C (1). However, IIF will only occur under these conditions when extracellular ice is present (1). Attempts to nucleate

a supercooled cytoplasm in the absence of extracellular ice have been unsuccessful. In all cases, the cell cytoplasm reached temperatures very close to the homogenous nucleation temperature of -40°C before nucleating (2.3). Further, investigations with liposomes, membranes with no intracellular components to act as nucleators, demonstrated IIF at temperatures above -10°C (9). The cell membrane has been shown to be an effective barrier to ice growth (1). If extracellular ice is an important element in the nucleation of the cytoplasm, then the plasma membrane must be involved. Work with nonacclimated and acclimated protoplasts has shown the effect that suspending medium as well as the compositional alterations of the plasma membrane during cold acclimation have on IIF (10,11,12). Similarly, work with hydrophilic antifreeze proteins has suggested that by promoting a closer interaction of ice with the plasma membrane the incidence of IIF can be affected (13,14). Presently there are three hypotheses which attempt to explain the mechanism by which extracellular ice interacts with the plasma membrane to initiate intracellular ice formation.

The first hypothesis is the pore theory proposed by Mazur in 1965 (1). This theory was motivated by the observations that supercooled cells could freeze internally well above the homogenous nucleation temperature of -40°C, and that the plasma membrane was an effective inhibitor of ice only above a certain temperature. He concluded that external ice could induce the supercooled cytoplasm by growing through aqueous pores in the membrane. In order for this to occur, the tip radius of the growing ice crystal must approximate the radius of the pores in the plasma membrane. The kelvin equation predicts that the smaller the radius of curvature of the ice crystal, the lower its melting point (15). Temperatures therefore exist where the crystal dimensions are insufficient to

allow for the propagation of ice through the pores of the membrane. Mazur proposed that as the cooling rate increases, the tip radius of a growing ice crystal approximates the radius in the plasma membrane and nucleation can result.

Disruption of plasma membrane has been proposed as an alternative mechanism by which extracellular ice can breach the cell. Working with protoplasts, Asahina proposed in 1962 that the cause of intracellular ice formation was the result of damage to the plasma membrane (16). The cold acclimation of protoplasts has been shown to stabilize the plasma membrane, and reduce the degree of IIF in these systems (10). Steponkus and Dowgert were able to observe ruptures in the membranes of plant protoplasts immediately prior to the formation of intracellular ice (17). This disruption has been proposed to occur as a result of the development of electrical transients created at the interface of the growing ice front and the aqueous solution (12). These electrical transients could cause the membrane to rupture if they reach a critical magnitude thus allowing ice from the extracellular solution to nucleate the cytoplasm. Motivated by the fact that cells can be damaged at 0°C in the absence of ice at a magnitude of osmotic pressure remarkably similar to those produced during freezing, Muldrew and McGann proposed that membrane damage may be a result of a critical osmotic pressure gradient across the membrane (18). While the osmotic pressure gradient itself may not be the damaging factor, the frictional forces developed as a result of the excessive efflux of water may result in rupture of the membrane (19). Disruption of the cell plasma membrane can therefore allow for the induction of extracellular ice into the cell.

The final hypothesis for the mechanism of intracellular ice formation in cellular systems suggests that the membrane of a cell can behave as a nucleator of

internal ice when acted upon by extracellular ice. This idea of surface-catalyzed heterogeneous nucleation was initially proposed by Toner in 1990 (20). The theory attributes the formation of intracellular ice to the ability of external ice to interact and alter the structure of the cell membrane. The nature of this interaction could be chemical, electrical, mechanical, ionic or thermal, but appears to make the plasma membrane an effective nucleator. The external ice can therefore induce the formation of intracellular ice without physically disrupting the integrity of the plasma membrane (20).

The three mechanisms proposed for the formation of intracellular ice each assumes a different role for the plasma membrane. In the pore theory, the cell membrane is an effective inhibitor of external ice only above the temperature at which the ice crystals are of insufficient size to propagate through the membrane pores. The membrane failure hypothesis requires the integrity of the membrane be complete disrupted and that cell damage precede the initiation of IIF. Finally, the surface-catalyzed nucleation theory suggests that the barrier properties of the cell membrane do not have to be compromised for the initiation of internal ice. While all of these theories propose alternative means by which ice can enter the cell, each one supports the assertion that it is the extracellular ice interacting with the plasma membrane that is responsible for the formation of intracellular ice.

Since the mechanism(s) of IIF is still speculative, the ability to predict the probability of IIF for a given freezing protocol has met with very limited success. However, the search for mathematical models which can predict the occurrence of IIF has been motivated by the potential use of these models in the design of cryopreservation protocols and their ability to further the investigation of the

mechanisms of IIF. The basis for all of the current models for the prediction of IIF have been based on Mazur's physiochemical model for water transport (4). Numerous phenomenological (11,21,22,23) models have been developed which use statistics and an understanding of the conditions surrounding the cell just prior to freezing to predict the likelihood of intracellular freezing. Mechanistic models have also been developed (18,20), which incorporate the mechanisms of membrane failure and surface-catalyzed nucleation to predict the occurrence of IIF. These two approaches, phenomenological and mechanistic, while fundamentally different in design, have been shown to give relatively similar end results (24). Each model is able to predict with some degree of certainty, the degree of intracellular ice formation in a cellular system under very controlled conditions.

The current tenet in cryobiology is that intracellular ice formation is lethal to cells. However, the degree to which intracellular ice is damaging to cells, and the mechanism by which this injury occurs has been largely speculative. The most widely held view is that IIF damage occurs as a result of mechanical damage due to recrystallization (5). Recrystallization occurs during slow warming, and is manifested as a net increase in the size of intracellular ice crystals. The observation that cells could be 'rescued' from intracellular ice formation as a result of rapid warming techniques (5), has provided support to this mechanism of damage. Recrystallization, however, is not the only means by which IIF can be lethal. Various non-mechanical mechanisms have also been proposed including: solution effects and thermal shock (25), osmotic injury (26), protein denaturation (27), and gas bubble formation (28,29,30). While the presence of intracellular ice

has been thought to result in irreversible damage to cells frozen rapidly, conclusive evidence to support this claim has been difficult to obtain.

Intracellular ice formation may not be in itself lethal. The fact that rapidly cooled cells can survive if rapidly warmed suggests that the ice itself is not damaging. Instead, the amount of ice, the size of the ice crystals (31), and the location of the ice formed (32,33,34) have been identified as conditions which when left unchecked, can lead to cellular damage. There have been numerous studies which suggest that innocuous intracellular ice formation is possible if the amount of ice formed can be controlled (35,36). Minimizing the size of the ice crystals formed during rapid cooling and warming to the point that the sample remains transparent, has been shown to be successful at preserving tumor cell function (37,38). Indeed, the fact that there are few morphological differences between rapidly frozen tissue and unfrozen tissues has allowed electron microscopists to use rapid cooling rates to minimize the degree of structural disruption. However, the amount of ice and the size of the ice crystals formed might not be as important as their location in the cell. Studies have shown that the nucleus of cells are more susceptible to the formation of intracellular ice (32,33). Disruption of the sensitive structures found inside the nuclear membrane would seriously affect the ability of a cell to function. By limiting the size of the ice crystals formed and protecting all of the sensitive sites in a cell, intracellular ice may be tolerated. If these conditions can be practically met, then the potential exists for novel approaches for the cryopreservation of cells. However, mainstream acceptance of the concept of innocuous intracellular ice formation needs occur first.

The development of intracellular ice in cells is traditionally detected by a sudden darkening of the cytoplasm. Termed 'flashing', this event corresponds with the

rapid formation of large ice crystals which are capable of scattering light. However if a cell is cooled rapidly enough, or has been sufficiently dehydrated prior to the formation of intracellular ice, than only small ice crystals will form. This type of IIF has been dubbed twitching (39). Techniques therefore exists for the differentiation of lethal and potentially innocuous forms of intracellular freezing.

The conditions used to induce IIF throughout this thesis resulted in the formation of large ice crystals, as detected by flashing. It will be assumed that this type of IIF results in lethal damage to the cell as a result of physical and chemical disruptions to the cells. However, the potential for non-lethal formation of intracellular ice as a result of rapid cooling and warming in the *in vitro* tissue models employed herein is not precluded.

1.4 TRADITIONAL CRYOPRESERVATION OF CELLS

Damage to cells can be caused by both intracellular freezing and exposure to high concentrations of solutes. The successful cryopreservation of a wide variety of cell types has been a result of the development of novel techniques to minimize both types of damage. All of the current protocols aimed at minimizing cell damage emphasize a reduction in the amount of ice formed. During slow cooling, a reduction in the ice formed can limit the concentration of extracellular solutes and hence the degree of damage. Similarly, during rapid cooling, if ice formation can be inhibited or limited, then the scale of damage done to the cells can be significantly reduced. Avoidance of ice formation has therefore been the focal point for the cryopreservation of cellular systems.

In 1949, Polge, Smith and Parkes introduced the idea of using chemical compounds to enhance the survival of frozen biological material. With the

addition of glycerol to their samples, they were able to demonstrate a significantly greater proportion of viable bovine spermatozoa after thawing from -79°C (40). A variety of chemical compounds, or cryoprotectants, have since been shown to colligatively effect the physical properties of solutions. Cryoprotectants are capable of reducing the amount of ice formed in a concentration-dependent manner. However, it quickly became known that high concentrations of cryoprotectants had detrimental toxic and osmotic effects (41,42) on cells. Innovative protocols for the addition and removal of these cryoprotectants were therefore developed in efforts to avoid their damaging effects when in high concentrations.

The effects of ice can be further limited if the formation of ice itself is completely avoided. Vitrification is the process by which a rapidly cooled solution bypasses ice formation and becomes an amorphous, glassy solid. By preventing the formation of a crystalline solid (ice), and the corresponding solute concentration, this method provide a means to significantly reduce the damage done to cells during freezing. However, in order to vitrify a sample, high concentrations of cryoprotectants and ultra-rapid cooling rates must be employed. In most cases, these conditions have prevented the successful vitrification of cells.

The traditional approach to the cryopreservation of cells has therefore been to balance the toxic effects of high concentrations of solutes with cooling rates slow enough to avoid IIF, but rapid enough to minimize exposure to the cryoprotectants. This method is based on the principle of preventing the formation of intracellular ice, and assumes that any intracellular ice is lethal to the cells. Through the extensive study of the intricate elements of a cells reaction to cooling and warming at different rates and under various conditions a

comprehensive understanding of the effects of low temperatures on cellular systems has been achieved. This information has facilitated numerous productive approaches for the cryopreservation of a wide variety of cell types.

1.5 TISSUES

Tissues are a complex system of organized cells. The organization of these cells can take many forms. In connective tissues, sparsely distributed cells are arranged in a elaborate extracellular matrix which together facilitates the maintenance of the tissues structure. Cartilage, as an example, is composed of cells (chondrocytes), which function to sustain the composition of an extracellular matrix (43). The cartilage matrix is composed of collagen and proteoglycans which confers the mechanical properties of the tissue (43,44). Alternatively, in epithelial tissues, cells are held together by direct cell-cell attachments, and there is only a thin extracellular matrix. The endothelial layer of the cornea is an example of a epithelial tissue (45). The hexagonal shaped endothelial cells of the cornea form a continuous monolayer that functions to maintain the degree of hydration of the corneal stroma. In corneal endothelium, the cell-cell connections support the mechanical stresses that may be imposed on the tissue. While the organization of cells is different in connective and epithelial tissue, there is a universal presence of junctions between adjacent cells and cells and their external environment. It is these junctions which makes tissues more than just the sum of their cellular components.

Cell-cell and cell-matrix interactions have been shown to be critical in a number of basic biological processes. Tissue structure maintenance, cell secretory activity, wound healing, morphogenic movement, cellular migration, and cell-cell communication all exist because of the presence of cell interactions. Cell

junctions can be classified into three functional groups: occluding junctions, anchoring junctions and communication junctions (46). Tight junctions (zonula occlundens) function to control the diffusion of molecules across the epithelium by acting as a barrier restricting the passage of molecules between cells, and by modulating the distribution of apical and basolateral membrane-bound carrier proteins. Anchoring junctions (zonula adherens junction, desmosomes and hemidesmosomes) connect the cytoskeleton of a cell to those of its neighbours or to the extracellular matrix. Finally, gap junctions allow for cell-cell communication by permitting small molecules to pass directly from cell to cell. Cell junctions play a critical role in the physiology of tissues.

1.6 REVIEW OF CRYOPRESERVATION OF TISSUES AND ORGANS

The widespread clinical application of cryopreservation to cellular systems during the past decade has created an environment in which the preservation of tissues for transplantation is highly desired. Tissue transplantation has the potential to significantly enhance the quality of life in patients. Whether it is the restoration of vision as a result of a cornea transplant, or a life-saving heart-valve replacement, the key to these treatments is the availability of the tissue. Long term storage of tissue as a result of cryopreservation would ensure tissue availability and would further provide a means for more effective donor-recipient matching, tissue typing and infectious disease testing, and national and international tissue sharing. Development of techniques for the low temperature and hypothermic storage of tissues is therefore a critical component in the evolution of transplant medicine (47).

Techniques for the cryopreservation of tissues have taken two distinct paths. The traditional approach has been to use high concentrations of cryoprotectant and

rapid cooling rates to reduce or eliminate intracellular ice formation. By balancing the toxicity of the cryoprotectants and osmotic stresses imposed during its addition and removal, vitrification of tissues and organs has been actively pursued. Recent reports of the vitrification of rabbit kidneys after exposure to 8M dimethyl sulfoxide (Me_2SO) and cooling to -32°C indicate that this technique may be feasible for the preservation of whole organs (48). However, there still exists numerous physical limitations to this technique which must be resolved (49). The second method for the cryopreservation of tissues involves the use of slow cooling and controlled equilibration of cryoprotectants coupled with rapid warming. This technique has been shown to be relatively successful in the preservation of arteries (50), split-thickness skin (51) and pancreatic islets (52). Under these conditions, thermal and mechanical effects become important factors in the practical application of these techniques (53).

Protocols for the cryopreservation of tissues and organs have largely been based upon models developed using the biophysical and physiological data collected from cellular systems. However, the complexity of organized tissues has made this approach a cumbersome and ineffective method to study the responses of tissues. The diversity of cell types and cell densities as well as their morphological differences complicates the determination of the osmotic and thermal state of the system (54). Detailed mathematically models have been developed which attempt to address these complex interacting variables. These models have included: the Krough cylinder model (55); the network thermodynamic model (56); the modified Krough cylinder model (57); and most recently the compartmental model (58). However, each of these models is limited by their ability to only predict what is known. Therefore a more detailed

understanding of the mechanism by which tissues are damaged would greatly enhance the efficacy of mathematical modeling.

Although successful cryopreservation can be achieved for a variety of cell types isolated in suspension, when *in situ*, these cells are severely damaged. For example, cells from the heart (59), the liver (60) and the kidney (61) can recover partial viability upon freezing while in suspensions. However, attempts to freeze these whole organs has proved unsuccessful (47,54). Additional factors must be affecting *in situ* cells ability to respond to low temperatures and the damage they incur. The ability to isolate and preserve cells from tissues, while being unable to preserve these same cells *in situ*, has begun to focus attention on the unique morphological and physiological characteristics of tissues.

The presence of cell junctions in multicellular systems has been proposed as a contributing factor to their enhanced sensitivity to freezing injury (34,62, 63,64,65). The mechanism by which damage occurs to tissues as a result of these cell-cell and cell-matrix interactions has been the subject of debate. Disruption of the cell junctions by the physical and chemical changes that transpire during freezing could affect the viability of the tissue. Formation of extracellular ice in tissues and organs has been shown to disrupt the structure of tissues and severely inhibit survival (34). The intercellular junctions have also been shown to be affected by exposure to the cryoprotectants (63) and osmotic stresses (62,64) encountered during freezing. In contrast, the cell junctions themselves might be the cause of a tissue's increased susceptibility to damage. Cell-cell and cell-surface interactions have been identified as conditions capable of modulating the prevalence and kinetics of intracellular ice formation in tissue

systems (65). As either the cause, or the result of damage, cell junctions have a distinct role in a tissues ability to respond favorably to low temperatures.

Of interest to this thesis is the mechanism by which cell junctions can facilitate an observed increase in intracellular ice formation. In their study in 1992, Larese *et al.* used a hamster fibroblast cell line that did not form any direct cell-cell attachments (65). However, anchoring to a substrate and the growth of a confluent monolayers were still shown to enhance the formation of intracellular ice in the cells. It has been suggested that the change in cell morphology as a result of attachment will predispose a cell to increased membrane damage as a result of the osmotic stress imposed during freezing (62,66). The role of cellular morphology on membrane damage in tissues during freezing will be explored in this thesis. It has further been reported that cell-cell contact can facilitate the nucleation of ice between adjacent cells (65,67.68,69,70,71). In these studies, the nucleation of one cell, after a brief delay, was followed by the freezing of an adjacent cell. Clearly, the capacity for a cell to induce ice formation in an adjoining cell will severely enhance the damage done to tissue during freezing. An understanding of the mechanisms by which this damage occurs, would greatly enhance the development of techniques for its minimization or complete avoidance.

A tissue is composed of multiple cells and multiple cell types that interact together to define the function of that tissue. This complex, coordinated interaction between the constituent cells, adjacent cells and the extracellular matrix has been identified as being important in the overall condition of the tissue. Our ability to successfully cryopreserve these complex systems will be significantly affected by our capacity to understand the interactions that exist in

tissues. Determining how these cell junctions are affected by excursions to low temperatures and effect the damage incurred by tissues during freezing will greatly enhance our understanding. This information will provide a clearer elucidation of the mechanism(s) responsible for the induction of ice between neighbours, and will facilitate the development of more accurate models that may assist in the cryopreservation of tissues.

1.7 HYPOTHESIS

Cell junctions have a distinct role in a tissue's ability to respond to low temperatures. The induction of intracellular ice in adjacent cells via cell-cell interactions contributes to the enhanced sensitivity of tissues to freezing.

1.8 OBJECTIVES AND APPROACH

The successful cryopreservation of tissues has been limited by the lack of information on the mechanism by which these systems are damaged as a result of exposure to low temperatures. The unique morphology and cell interactions characteristic of tissues has been proposed as contributory factor to their enhanced sensitivity to freezing injury (63,64,66). The formation of intracellular ice, a potentially lethal form of cryoinjury, has been shown to occur more predominantly in biological systems that possess cell-cell and cell-surface interactions (65,68,69). This thesis will explore the effects that cell junctions have on the mechanism of intracellular ice formation in tissues.

In order to prove the hypothesis, it will be necessary to show that a direct correlation exists between the presence of cell junctions and the incidence of intracellular ice formation in tissues. By establishing this relationship it will then be possible to investigate the mechanism by which cell adhesions are capable of effecting the formation of ice in tissues. Three studies have been designed to

investigate the mechanism by which intracellular ice formation forms in tissue systems, and the means by which this damage affects a tissue's ability to respond to low temperatures.

1. ***To elucidate the role of cell junctions on the membrane integrity and incidence of intracellular ice formation.***

   The plasma membrane is the most important cellular structure from the perspective of low temperature biology. As the primary point of contact between the cell and its environment, the cell membrane must be able to react to the physical and chemical changes that occur as ice forms extracellularly. The surface area it encompasses and its permeability to water and cryoprotectants have been demonstrated to be important determinants of a cell's response to low temperatures. Similarly, the plasma membrane has been identified as a critical element in the initiation of intracellular ice formation in cells. The maintenance of the cell's membrane integrity is therefore critical to its ability to survive freezing.

   Experiments will be conducted to investigate the possibility that cells *in situ* are more susceptible to membrane damage and subsequent intracellular nucleation because of their unique morphology. Cell-cell and cell-surface interactions have been identified as important modulators of the systems response to low temperatures (63,64,65,66). Three model systems with defined degrees of cell-cell and cell-surface connections will be utilized. Coupling cryomicroscopy with fluorescent dye exclusion assays, the incidence and patterning of IIF will be correlated with the integrity of the cell plasma membrane. Using this technique, the effects of cell junctions on the

membrane integrity and incidence of intracellular ice formation will be determined.

2. ***To determine the role of cell-cell contact on the incidence and pattern of intracellular ice formation.***

   Previous work has suggested there exists a pattern in the formation of IIF in tissue monolayers (65). This observation has raised the question of how intracellular ice is formed in tissues. Using the cryomicroscope in conjunction with real-time and video assessment, the relationship between IIF in cells in relative proximity to one another will be explored. Pattern recognition programs will be utilized to chart this relationship, and with statistical analysis, the influence of cell-cell contact on intracellular ice formation will be determined. This technique will allow for the consideration of the possibility that ice induction between adjacent cells is responsible for the observed increased incidence of IIF. Statements can then be made on the potential mechanism by which this may occur.

3. ***To monitor the effects that gap junctions have on the patterning of IIF in tissues.***

   The presence of gap junctions in cell monolayers allows small molecules to pass through the plasma membrane of two adjacent cells. It has been suggested that these unique structures could provide a means by which intracellular ice can propagate between adjoining cells (68). Using two different cell lines, one of which forms gap junctions, the effect of these communication pathways on the incidence and distribution of intracellular ice formation will be determined.

The lethal injury that is attributed to the formation of intracellular ice and its increased prevalence in tissues, demands that a more detailed study of the environment surrounding its genesis be undertaken. This thesis will explore the role of cell junctions on the induction of intracellular ice in adjacent cells.

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CHAPTER 2
ROLE OF CELL CONTACT ON MEMBRANE INTEGRITY AFTER INTRACELLULAR ICE FORMATION

2.1 INTRODUCTION

The cryopreservation of tissues for transplantation has met with limited success as a result of a lack of understanding of the mechanisms responsible for increased tissue damage during freezing. Current techniques for the preservation of tissues are primarily modifications to the cryopreservation protocols developed for cellular systems. Despite prolonged efforts at continued empirical modification of these methods, the cryopreservation of tissues still remains limited to a select few tissues. Among those tissues now routinely preserved, it is evident that an improvement in their quality is required to meet increasing clinical demands. With post-thaw viability of these tissues being one of the major limiting factors in successful transplantation, a better understanding of the mechanisms of injury in these systems needs to be developed. Further, a clearer understanding of the biochemical and physiological aspects of tissue function will provide a better base of knowledge from which effective protocols for tissue cryopreservation can emerge.

Although successful cryopreservation can be achieved for a variety of cell types isolated in suspension, when *in situ*, these cells are severely damaged during freezing. For example, cells from the heart (1), the liver (14), the cornea (2), and the kidney (9) can all recover partial viability after freezing while in suspension. However, attempts to freeze these whole tissues and organs has proved unsuccessful (5,6,7). It is likely that additional factors are affecting the low temperature responses of cells *in situ*, and causing the damage incurred. The

ability to isolate and cryopreserve cells from tissues, while being unable to cryopreserve these same cells *in situ*, is focusing attention on the cryophysiology of tissues. In 1979, Pegg *et al.* postulated on the reasons for increased susceptibility of organs and tissues to cryoinjury. These included fixed geometry, cell density, diversity of cell types, and vascularization (6). Tissues and organs are more than just a mass of cells, and the structural organization and cellular differentiation unique to these systems likely plays an important role in their capacity to respond to low temperatures.

The response of cells to freezing is critically dependent on the presence of an intact cell membrane. As the primary area of contact between the cell and its environment, the cell membrane must be able to react to the physical and chemical changes that occur as ice forms extracellularly. A severely damaged plasma membrane has been shown to be a practical indicator of the occurrence of lethal injury to a cell (8,9,10). As a barrier to ice propagation and the sole surface for the permeation of water and extracellular solutes, the plasma membrane performs a precise role in the low temperature responses of cells.

During cooling, if the cell is maintained near normal physiological conditions through the exchange of solutes and water across the membrane, then relatively little damage is incurred. However, if the cell is unable to maintain equilibrium with the external environment then intracellular ice formation will result. For IIF to occur two conditions must be met: the cell cytoplasm must become supercooled, and a nucleation event must occur. Supercooling has been shown to occur as a result of an imbalance in the osmotic gradients that develop across the plasma membrane during rapid cooling (11). However, the means by which the cytoplasm becomes nucleated has been the subject of much debate. There is

strong evidence to suggest that extracellular ice and the plasma membrane are involved in the initiation of IIF. As the cell plasma membrane is an effective barrier to the propagation of ice (12), this barrier must be breached for nucleation to occur. The interaction of extracellular ice with the membrane has therefore been a consistent theme in current theories on the mechanism of intracellular ice formation in cellular systems (12,13,14,15,16).

Tissues are a complex system of organized cells that maintain their structure and function through the development of cell junctions. Using *in vitro* tissue models, cell-cell interactions have been identified as important determinants of a tissue's response to cryopreservation. Larese *et al* characterized the intracellular ice formation (IIF) behavior of hamster fibroblasts in various morphological states that simulated some of the structures found in organized tissues, and identified cell-cell contact as a mediator of the prevalence and kinetics of IIF (17). The development of cell junctions between cells and the organization of the cytoskeleton have been shown to further render cells more sensitive to injury at subzero temperatures (18,19). These close interactions which characterize most tissue systems and which are essential for tissue function, now appear to be likely sites for tissue damage by osmotic stresses (20) and phase changes (21) involved in the process of cryopreservation. This underlines the need for a better understanding of the effects of cell-cell interactions on a tissues response to low temperature.

In 1975, McGrath and Cravalho used a fluorescent viability test to correlate membrane integrity with cell damage during slow and rapid cooling (22), and reported that the proportion of cells forming intracellular ice was greater at a given cooling rate for a sample which had a higher cell concentration. Other

effects of cell packing on the low temperature response of cellular systems have been previously reported (23,24,25). Although the influence of the extracellular salt concentration and the unfrozen fraction has been identified as important parameters in explaining the affect of cell concentration on overall survival, the mechanism by which cell contact can intensify damage is still unknown (23). A detailed examination of the role that this cell-cell contact has on the membrane integrity and susceptibility of cells *in situ* to form intracellular ice would facilitate a better understanding of a tissues response to low temperatures.

The objective of this study is to investigate the role of cell-cell contact on the formation of intracellular ice and subsequent effects on the integrity of the cell plasma membrane. Three cell models with defined degrees of cell-cell and cell-surface interactions were used to investigate the capacity of cell junctions to modulate the incidence of intracellular ice formation.

2.2 MATERIALS AND METHODS

Cell Culture

All cells used were derived from the V-79W line of Chinese hamster fibroblasts. The cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential medium (MEM) with two-percent 7.5% (v/v) sodium bicarbonate solution, 1% (v/v) L-glutamine and 10% (v/v) fetal bovine serum supplemented with antibiotics (penicillin G (100 µg/ml), streptomycin (100 µg/ml)) (all components from GIBCO Laboratories, Grand Island, NY). Cells were kept in tissue culture flasks (25 cm^2; Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C. The fibroblasts were re-suspended in supplemented MEM. For cells in a confluent monolayer, sterilized cover slips (12 mm circle, FISHER Brand) were placed in a

petri dish (FISHER Brand, 100x15 mm) and covered with 15 ml of supplemented MEM containing $1x10^5$ cells. The petri dishes were kept in an incubator for 12 h to allow the cells to attached and 5 d to allow the growth of a monolayer.

The three *in vitro* model systems investigated were cells in suspension, single cells attached to glass and confluent cell monolayers. Cell suspensions are characterized by individuals cells dispersed in a suspending media. In this configuration, there are no cell-cell or cell-surface interactions at the low cell densities considered. Using the natural adherence of fibroblasts, single cells attached to a glass substrate permitted the analysis of the effects of cell-surface interactions. Binding to a substrate results in cells assuming a flat, non-spherical shape where one face is excluded from exchange with the extracellular medium. Confluent monolayers result when cells attach and divide forming both cell-surface and cell-cell interactions. The V-79W fibroblasts do not form gap junctions between adjacent cells.

Cryomicroscope and Video System

The cryomicroscope and video system used for this study consisted of a Zeiss light microscope (Carl Zeiss, Germany), a CCD video camera (ZVS-47DEC, Carl Zeiss, Germany), a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar to the design described by Diller *et al.* (26,27). The base of the cryostage consisted of a four-inch square block of ¼ inch teflon. This material was chosen because of its low coefficient of thermal conductivity and the ease with which it can be obtained and worked. A ¾ inch cylinder was removed from the center of the base to allow for the passage of light and to create a nitrogen chamber. Two intake and exhaust ports allow dry nitrogen gas to enter and exit the chamber. Two glass coverslips sealed the chamber and provided a surface

for the experimental sample to be cooled by the nitrogen gas. A temperature control system consisting of a transparent metal oxide coating on the bottom surface of the upper slide, a 0.005 inch copper-constantan thermocouple (OMEGA Engineering, Inc., Stamford, CT) and a computer-controlled interface (GCCC, Spruce Grove, AB). The computer monitored the temperature by analyzing the voltage from the thermocouple and via a proportional controller circuit, heat was added as necessary to allow the stage to follow a user defined thermal protocol. A schematic of the cryostage used in this study is shown in figure 2.1.

Membrane Integrity Assay

A dual fluorescent staining technique was used for the quantitative assessment of the integrity of the cell plasma membrane. SYTO, a live cell nuclear dye (Molecular Probes, Eugene, OR), and ethidium bromide (EB; SIGMA Chemical Company, Mississuaga, ON) where used to differentially stain the cells. SYTO acts by entering intact cells and labeling both RNA and DNA with a uv excited green emission. EB has been shown to penetrate only cells with damaged membranes and form a complex with nuclear DNA (28). The red EB fluorescence is more pronounced than the green SYTO fluorescence so cells with intact membranes fluoresce green, and cells with damaged membranes fluoresce red. The SYTO / EB assay for membrane integrity has been previously shown to correlate directly with the overall viability of the cell post-thaw (19). This is consistent with other dual florescence staining techniques which have been used in the assessment of cell viability (8,9)

Ethidium bromide was stored at 4°C as a 25 μM stock solution in phosphate buffered saline (PBS; GIBCO Laboratories, Grand Island, NY). A working

solution of ethidium bromide was prepared by diluting the stock solution 1 to 10 in supplemented MEM. The SYTO was prepared as a 12.5 µM stock solution in PBS and stored frozen at -20°C. The SYTO/EB assay solution was prepared daily by adding 100 µl of ethidium bromide working solution and 10 µl of SYTO stock solution to 1 ml of supplemented MEM. The final concentration of SYTO was 1.25 µM and of EB was 2.5 µM. Single cells attached to glass and cell monolayers were stained with 100 µl/coverslip. A concentration of 100 µl/ml was used for cells in suspension. Samples were examined under a fluorescent microscope using a mixture of brightfield and ultraviolet illumination (440-480 nm) at different stages of the freezing process.

Detection of Intracellular Ice

The formation of intracellular ice results in the scattering of light. The traditional method for the detection of IIF has been the occurrence of a sudden darkening of the cytoplasm (22,29). However, when monolayers are assayed for the formation of intracellular ice, particularly at lower temperatures, the high proportion of cells displaying this 'flashing' makes it difficult to quantify the IIF status of each cell in the monolayer. While the 'flashing' technique is effective for the cell suspensions and single attached cells, a novel approach for the detection of IIF in the cell monolayers was required.

Experimental Procedure

Cells in suspension, single cells attached to glass, and cell monolayers were stained with SYTO and EB and allowed to incubate at 22°C for 2 min. This was previously determined to be sufficient time to allow for the diffusion of the stains. A 7 µl sample of the cell suspension was placed on the cryostage and protected with a circular glass coverslip. Alternatively, the coverslips containing single

attached cells or cell monolayers where placed inverted on the cryostage after staining. The thermal protocol and image segments taped are illustrated in figure 2.2. The cryostage was cooled at -25°C/min to a predetermined subzero temperature where it was held for 10 sec. The sample was then nucleated at the constant experimental temperature using a cold copper probe, and held at the experimental temperature for 2 min. The sample was then warmed at 25°C/min to 37°C and held for 5 min. The entire procedure was videotaped, alternating between brightfield and fluorescent images, for later analysis. Still images were captured from videotape using a TARGA+ image capture system (Truevision, Indianapolis, IN) and analyzed using custom designed software. The exact location of each cell was facilitated by the nuclear-binding SYTO stain. This allowed for retrospective analysis of the data frame by frame to determine the incidence of intracellular ice formation. The addition of SYTO and EB to the experimental samples does not affect the incidence of intracellular ice formation in the three model systems (unpublished data).

Three samples were collected at each temperature with an average of 20 cells per sample for cell suspensions, 10 cells per sample for single attached cells and 150 cells per sample for confluent monolayers. The incidence of intracellular ice formation and EB positive cells denotes the percentage of total cells that display IIF or become damaged and score EB positive. Data points represent the average value with standard error bars.

2.3 RESULTS

Intracellular Ice Formation at Constant Temperature

Figure 2.3 shows the cumulative incidence of intracellular ice formation as a function of the temperature of nucleation for cell suspensions, single attached

cells, and cell monolayers. The temperature for 50% IIF ($^{50}T_{IIF}$) was determined from logistic curves fit to the data points. The values of $^{50}T_{IIF}$ are -9.4°C for cells in suspension, -8.0°C for single attached cells and -7.3°C for cell monolayers. This data is statistically similar to the data presented by Larese *et al.* ($P<0.001$; student's t test) and supports the observation that cell-cell contact facilitates intracellular nucleation (17).

Detection of Intracellular Ice Formation

The scattering of light due to the formation of intracellular ice is not only visible under brightfield illumination, but has also been reported using fluorescent illumination. In the presence of a fluorescent dye, a dramatic change in the intensity of the stain occurs when ice forms (22). However, the difficulty in applying this technique to tissue systems is similar to the 'flashing' assay. The cytosolic fluorescent stain used, fluorescein diacetate (FDA), stains the entire cell and the monolayer appears as a green continuum. When intracellular ice forms, the complete cell undergoes this intensity shift as a result of the scattering of the excitation light. At low temperatures the large number of cells that display this intensity shift makes it difficult to quantify IIF in individual cells. However, the use of a nuclear fluorescent stain (SYTO) permits identification of individual cells undergoing IIF as the fluorescence and intensity shift are restricted to the nucleus of each cell. The formation of ice in the nucleus disrupts the structures stained by the SYTO dye and results in a distinctive change in the fluorescent pattern (figure 2.4).

In order to assess the effectiveness of using the SYTO stain and CCD camera in detecting intracellular ice formation in cell monolayers, the cumulative incidence of IIF as a function of the temperature of nucleation for each detection method

was determined. There was no statistical difference in the detection of IIF between the two methods.

Membrane Integrity After Thawing

A comparison of the cumulative incidence of cells with a loss of membrane integrity and the cumulative incidence of IIF as a function of temperature is shown in figure 2.5. The results for the cell suspensions (figure 2.5a) and single attached cells (figure 2.5b) show that there is no significant difference in the number of cells that form intracellular ice and those that lose membrane integrity. However, figure 2.5c demonstrates that in cell monolayers there is a significant difference in the number of cells with intracellular ice and the number scoring EB positive particularly at lower temperatures. At -9°C where all of the cells display intracellular ice, only about 20% of the cells score EB positive. Intracellular ice formation does not lead to the immediate disruption of the plasma membrane in the majority of cells in a monolayer.

2.4 DISCUSSION

This study examined the influence of cell contact on membrane integrity and susceptibility of cells to the formation of intracellular ice. IIF in cell suspensions and single attached cells corresponded directly with an immediate loss in the integrity of the cell membrane upon warming. This supports previous work demonstrating the direct correlation of damage to the plasma membrane with conditions leading to intracellular freezing (14,22,30,31,32). In cell monolayers, however, only a small percentage of the cells displayed a loss of membrane integrity, yet a significantly greater proportion of the cells formed intracellular ice. The formation of intracellular ice in tissue systems is, therefore not an immediate cause or result of damage to the cell plasma membrane.

The decreased incidence of EB positive cells in cell monolayers may be a result of an inability for the dye to completely diffuse across the cell membrane as a result of a reduction in the available surface area for diffusion, or insufficient elapsed time for the influx of the dye. If a reduction in the surface area were to inhibit the movement of the dye, a similar dissociation between the prevalence of IIF and EB positive cells in the single attached cell model would be expected. The reduction in a monolayer's exposed surface area as a result of cell-cell interactions is considered to be relatively negligible. The rate of dye influx is not likely limiting in this study. Upon thawing, the influx of the ethidium bromide as evident by a change in the cell's color from green to orange to red, occurs rapidly. For all three of the model systems investigated, the cells that would demonstrate a loss in membrane integrity would completely undergo this color shift prior to the sample reaching 37°C. McGrath and Cravalho similarly observed a dramatic and immediate loss of the fluorescent dye in cells that formed intracellular ice (22). The diffusion of the ethidium bromide into monolayers does not seem to be a limiting factor in these experiments.

Cell-cell interactions can significantly effect a monolayer's ability to respond to low temperatures. The observed increase in the cumulative incidence of IIF in single attached cells and monolayers compared to cell suspensions implies that the physical morphology of the cell and the unique structures it forms during attachment and growth to confluency are able to increase the susceptibility to the formation of intracellular ice.

One recent hypothesis on the genesis of IIF proposes that the frictional drag of osmotically driven water can lead to a rupture of the plasma membrane which would allow extracellular ice to propagate into the cytoplasm (13,14). Scanning

electron microscopy has clearly demonstrated the extended morphology that fibroblasts maintain when attached to glass (17,34). During osmotic shrinkage, tension at local adhesions of the cell to glass may render the membrane more fragile to rupture, resulting in a higher incidence of IIF under these conditions. Another theory proposes that intracellular ice is catalyzed by the plasma membrane (16), where deformations in the presence of extracellular ice increases the contact angle of the plasma membrane which makes nucleation more likely. It is possible that the contact angle of the membrane in cells attached to glass provides a more effective nucleator for IIF than cells in suspension.

It has been reported that cell-cell contact can facilitate the nucleation of intracellular ice between adjacent cells (17,18,35). The nucleation of one cell, after a brief delay, is followed by the freezing of an adjacent cell. The ability of cells to interact with each other supports the observed increase in the prevalence of intracellular ice formation in tissues. However, the role that the membrane has in this induction mechanism is difficult to surmise. It is known that the cell-cell interactions that characterize epithelial tissues can be divided into three categories: occluding junctions, anchoring junctions and communication junctions. The later of these junctions, typically known as gap junctions, permit the passage of small molecules directly from one cell to another. It has been suggested that the induction of ice between adjacent cells could occur as a result of these gap junctions (18). If this were the case, then intracellular ice could form in monolayers as a result of the induction of ice between adjacent cells without the disruption of the cell membrane. While this may be the case in some epithelial tissues, the V-79W fibroblast cells used in this study do not form

intercellular communication junctions and therefore, the ability for ice to induce neighbouring cells must occur by an alternative means.

Since damage to the plasma membrane in tissue systems is not an immediate cause or result of intracellular freezing, the potential exists for the development of cryopreservation protocols which may minimize the damage resulting from IIF. Let us assume that lethal injury occurs to cells as a result of both membrane damage and the formation of intracellular ice. This is not an unrealistic assumption as the role of the membrane in the formation of intracellular ice has been well documented (12,14,16,30,36). By limiting the damage done to the membrane, as the case seems to be for tissue systems, the opportunity exists to reduce the overall injury cause by freezing. Observations of innocuous IIF have proposed that the damage cause by IIF is likely a result of the amount (37,38) and location of the ice formation (33,39,40) and not the ice itself. This is supported by the ability to rescue cells that have formed intracellular ice by rapid warming (41). The use of rapid cooling to minimize the size of the intracellular ice crystals formed, coupled with rapid warming rates to limit the recrystallization of these crystals, is not a novel cryopreservation technique. However, its application in tissue cryopreservation has been largely overlooked because of the view that the formation of any intracellular or interstitial ice is lethal.

The closely packed cells of tissues makes it difficult to assay IIF. An alternative technique uses the fluorescent dye SYTO, in combination with a low-light CCD camera to differentially determine the formation of intracellular ice. This method has been demonstrated to be as effective as the traditional flashing method for the visualization of IIF in cells *in situ*. The fluorometric assessment, however, is much more practical a technique than the 'flashing' method as data can be more

quickly gathered either by hand or in conjunction with automated software. This method also has the potential to provide a unique tool to probe the damage caused by intracellular ice. The crystalline surfaces that occur as a result of ice formation, result in the disruption of the pattern of staining in the form of 'pockets'. During warming, these 'pockets' of ice can be clearly seen to grow and then shrink due to recrystallization and melting. This technique, therefore, provides a more dynamic alternative to the freeze-substitution and electron microscopy experiments that have been commonly used to map the damage caused by the formation of intracellular ice (33,40). If the amount and location of ice formation is an important factor in the cryopreservation of tissues, development of this fluorometric method will serve as an innovative technique for future investigations.

Tissues are a complex arrangements of multiple cell types whose interaction together dictates their overall function. Freezing induces changes to these interactions, and in some cases can be amplified by these interactions, resulting in damage and lethal injury to tissues. Our ability to cryopreserve tissues will depend on our elucidation of the mechanisms by which the chemical and physical disruptions manifested during freezing affect their biological and biochemical components. The cryophysiology of tissues must be carefully studied if the successful cryopreservation of these complex systems it to be realized.

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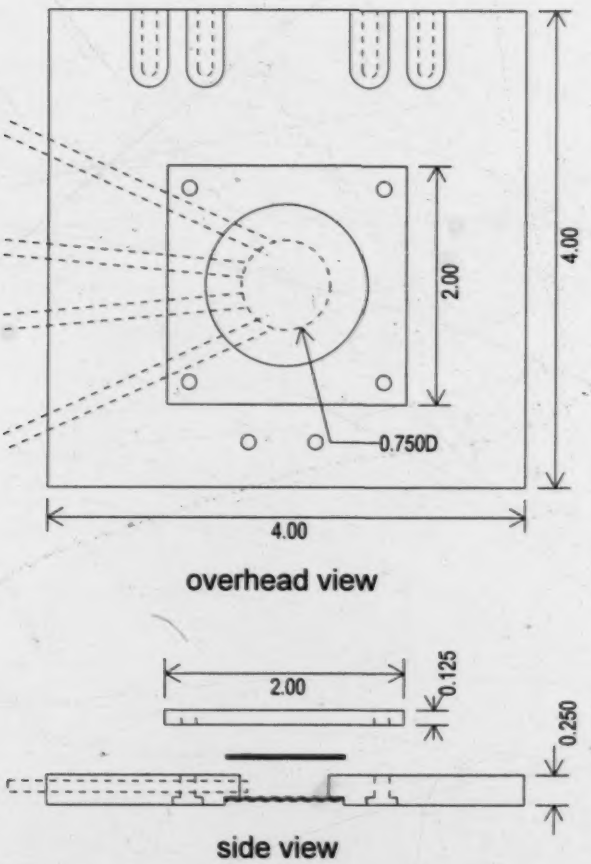

Figure 2.1 Schematic diagram of the cryomicroscope used in this study. The sample is cooled by convection from the nitrogen gas and warmed by the passage of an electrical current across the metal oxide coating . Units are given in inches.

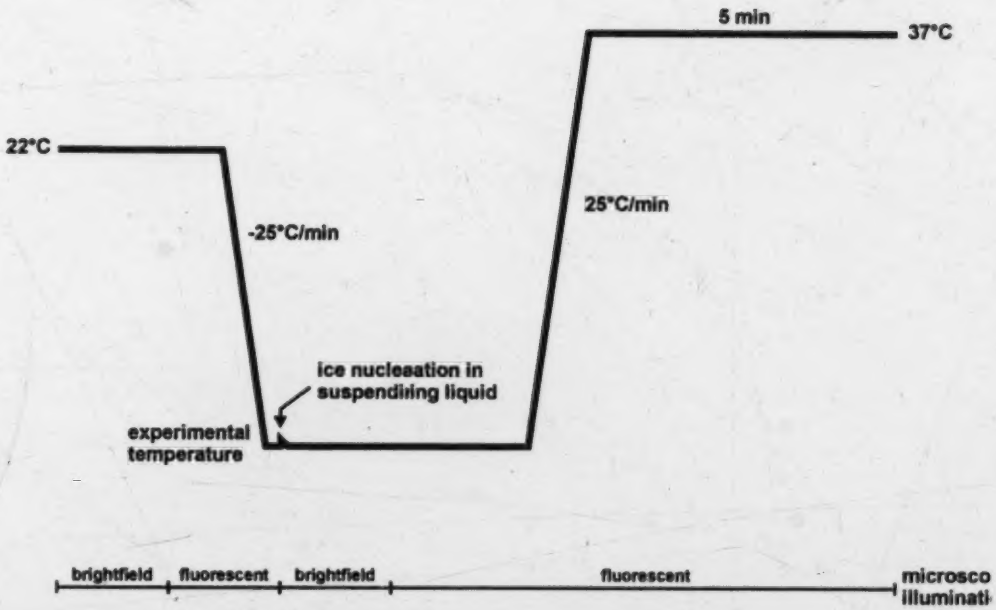

Figure 2.2 Cryomicroscope thermal protocol used for the constant cooling and warming of the three experimental *in vitro* tissue models.

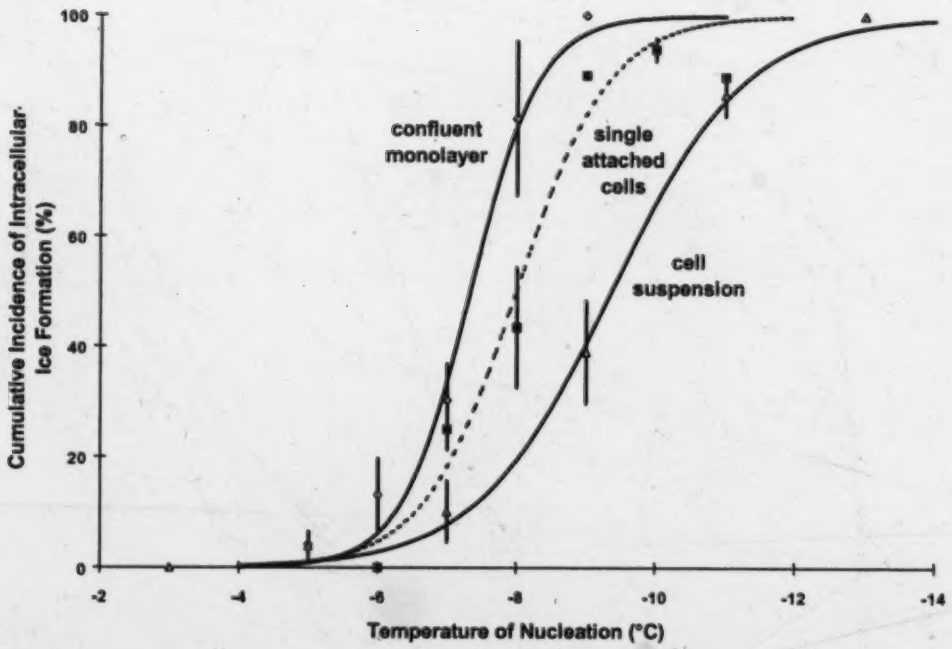

Figure 2.3 Cumulative incidence of intracellular ice formation as a function of temperature for the three experimental models: cell suspensions (open triangles); single cells attached to glass (closed squares) and confluent monolayers (open diamonds). Lines are logistic curves fit to the experimental data.

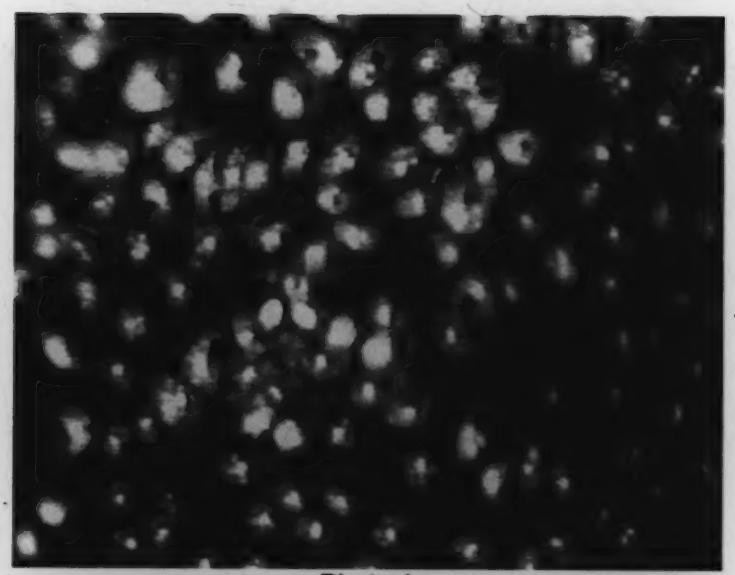

Photo 1

Photo 2

Figure 2.4 Photo 1 shows a V-79W hamster fibroblast monolayer stained with the fluorescent nuclear dye SYTO prior to freezing. Photo 2 shows the same monolayer after cooling at -25°C/min to -9°C. The formation of intracellular ice in the nucleus has disrupted the structures stained by the SYTO dye and results in a distinctive change in the fluorescent pattern.

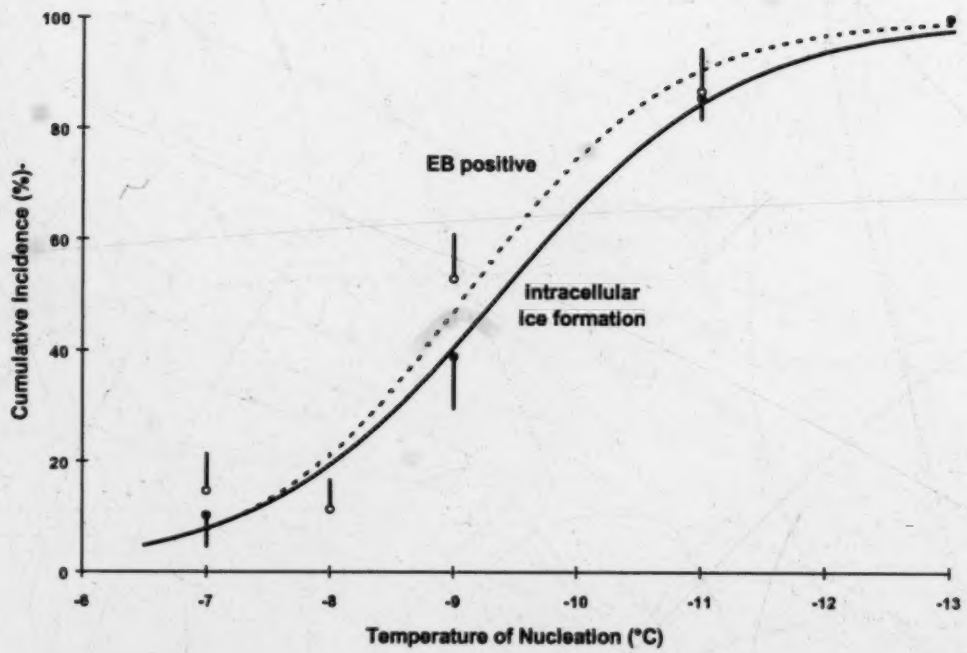

Figure 2.5a Cell suspensions - Cumulative incidence of intracellular ice formation (closed circles, solid line) and cumulative incidence of ethidium bromide positive cells (open circles, dashed line) as a function of temperature for V-79W hamster fibroblasts.

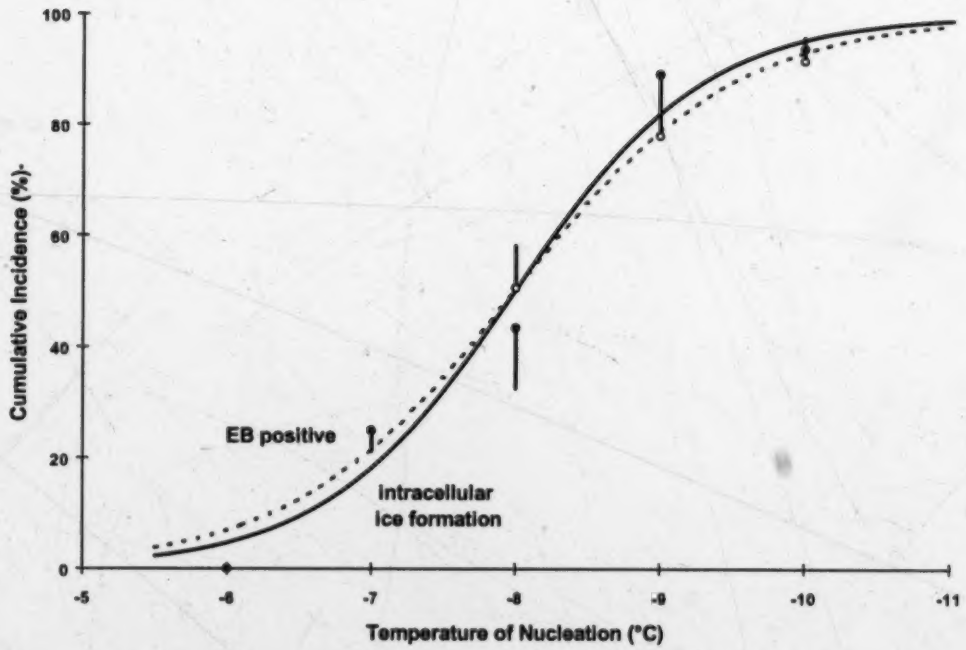

Figure 2.5b Single cells attached to glass - Cumulative incidence of intracellular ice formation (closed circle, solid line) and cumulative incidence of ethidium bromide positive cells (open circles, dashed line) as a function of temperature for V-79W hamster fibroblasts.

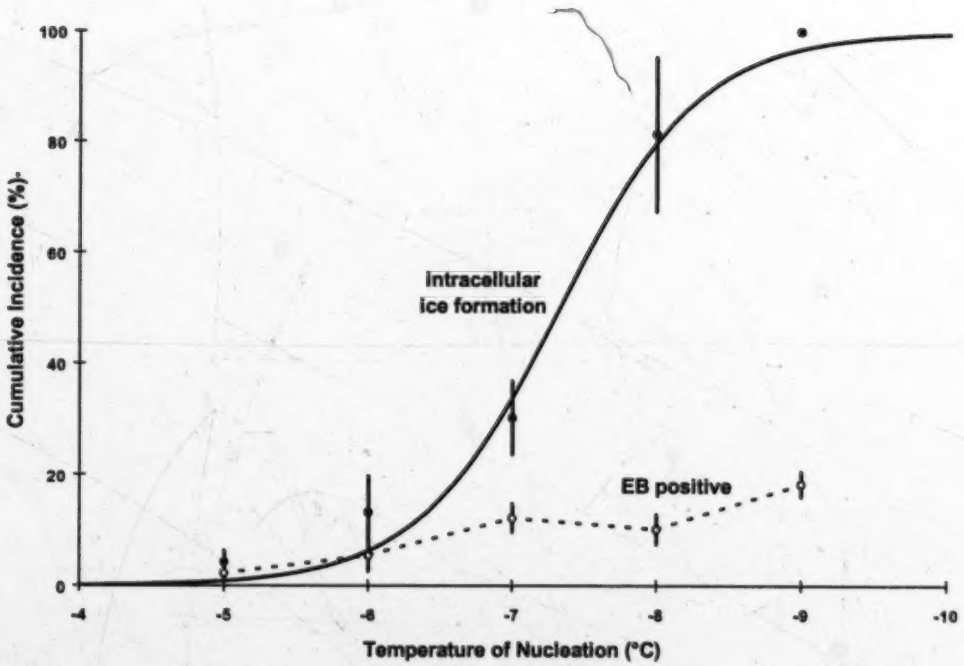

Figure 2.5c Cells in confluent monolayers - Cumulative incidence of intracellular ice formation (closed circle, solid line) and cumulative incidence of ethidium bromide positive cells (open circles, dashed line) as a function of temperature for V-79W hamster fibroblasts.

CHAPTER 3
THE ROLE OF CELL-CELL CONTACT ON INTRACELLULAR ICE FORMATION

3.1 INTRODUCTION

A tissue is composed of multiple cells and multiple cell types with complex, coordinated interactions between the constituent cells, adjacent cells and the extracellular matrix. These interactions determine the overall function of the tissue. As cell death occurs when the cell is unable to regulate its internal environment, lethal tissue injury occurs when cell contacts are not managed in a manner compatible with tissue function (1). These unique morphological and physiological characteristics have also been determined to be important in the low temperature response and subsequent post-thaw viability of tissues (2,3,4). The ability to successfully cryopreserve these complex systems, will therefore be significantly enhanced by a clearer understanding of the mechanisms by which cell-cell and cell-matrix facilitate damage in tissues.

During cooling, if the cell environment is able to be maintained at physiological levels through the exchange of solutes and water across the membrane, then relatively little damage is normally incurred. However, if the cell is unable to maintain equilibrium with the external environment then intracellular ice formation will result. Although the formation of intracellular ice is normally considered to be a lethal event, the mechanisms of damage are unclear. IIF may be the cause of (5,6,7,8) or result in (9,10,11) damage to cellular components. The difficulty in elucidating the means by which IIF forms and the mechanism by which it damages cells, has hindered the development of techniques for the cryopreservation of tissues. A current approach uses high concentrations of

cryoprotectant and rapid cooling rates. This method is based on the principle of preventing the formation of intracellular ice, and assumes that any intracellular ice is lethal. However, the critical cooling rate for tissue cryopreservation is significantly less than the cooling rate for the same cells in suspension (12), and will subsequently limit the effectiveness of rapid cooling protocols. In addition, the incidence of intracellular ice formation is considerably higher in tissues, than in cell suspensions or single cells attached to glass (13). It is critical for the advancement of tissue cryopreservation that the events surrounding the formation of intracellular ice be determined.

The presence of cell junctions in multicellular systems has been proposed as a contributing factor to their enhanced sensitivity to freezing injury. Disruption of the cell junctions by the physical and chemical changes that occur during freezing affect tissue viability. The formation of interstitial ice has been shown to disrupt the structure of tissues and severely inhibit survival (14). The intercellular junctions have also been shown to be affected by exposure to the cryoprotectants (2) and osmotic stresses (3,15) encounter during freezing. In contrast, the cell junctions can directly enhance the damage that tissues incur during freezing (13). It has been reported that cell-cell contact can facilitate the nucleation of intracellular ice between adjacent cells (13,16). This phenomenon was first documented by individuals investigating the freezing of plant tissue (17,18,19,20). They observed that the nucleation of one cell, after a brief delay, is followed by the freezing of an adjacent cell. By facilitating the nucleation of ice amongst adjoining cells, intercellular contact can enhance the incidence of intracellular ice in tissues.

The objective of this study is to investigate the effects that cell adjacency has on the incidence and kinetics of intracellular ice formation in a confluent monolayer. Using the cryomicroscope in conjunction with image and statistical analysis, an examination of the relationship between IIF in cells in relative proximity to one another will be conducted. An understanding of how this proximity affects the prevalence and distribution of ice in a tissue model system will provide a more quantitative method to assay the interactions between adjacent cells and will facilitate the clarification of the mechanism(s) by which ice forms in tissues.

3.2 MATERIALS AND METHODS

Cell Culture

This study used the V-79W Chinese hamster fibroblast cell line. The culture technique for these cells is described elsewhere (chapter 2). Briefly, hamster fibroblast cells were maintained in culture, seeded at 6.7×10^3 cells/ml and incubated for 5 d at 37°C to form confluent monolayers on coverslips.

Freezing at Constant Subzero Temperatures

The cryomicroscope used for this study is described in detail elsewhere (chapter 2). Briefly, it consisted of a Zeiss light microscope (Carl Zeiss, Germany), a low-light CCD video camera (ZVS-47DEC, Carl Zeiss, Germany), a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar to the stage described by Diller (21,22). A computer interface and program (GCCC, Spruce Grove, AB) was used to monitor the temperature and provide an output for proportional control of the heating element. The cultured cell monolayer attached to glass were stained with 1.25 μM SYTO stain (Molecular Probes, Eugene, OR) and allowed to incubated at 22°C for 2 min. The addition of the SYTO stain does not affect the incidence of intracellular ice formation in V-79W fibroblasts

(unpublished data). A 7 µl sample of the cell suspension was then placed on the cryostage and protected with a circular glass coverslip. Alternatively, the coverslips containing the cell monolayers were placed inverted onto the cryostage. The cryostage was cooled at -25°C/min to a predetermined experimental temperature and held at for 10 s. The sample was then nucleated at the constant temperature using a cold copper probe. The entire process was videotaped for later analysis.

Image Analysis

Still images were captured from videotape using a TARGA+ image capture system (Truevision, Indianapolis, IN) and analyzed using custom software. For each sample frozen at a constant temperature, both brightfield and fluorescent images were recorded from the unfrozen and frozen sample. The occurrence of intracellular freezing was assayed by a sudden darkening of the cytoplasm (23,24) in conjunction with the disruption of the SYTO stain (chapter 2).

Statistical Analysis

A statistical method was developed to investigate the effects of cell-cell adhesion on the induction of ice between adjacent cells. Using custom designed software, the fluorescent images were analyzed to determine the cells location in the x, y coordinate plane. The data was plotted and correlated with the presence of intracellular ice. Directly adjacent cells which displayed intracellular freezing were connected on the graph by a line (figure 3.1). This process was repeated for all of the temperatures investigated at equal time intervals, and each data point was normalized by dividing the number of connected components by the total number of cells that displayed IIF. The normalized number of connected components is a reflection of the total number of cells with IIF that are adjacent to an already

frozen cell. This number will be the test statistic. A randomly generated frequency distribution was devised by calculating the number of connected components in a purely random sample. As our test statistic is discrete, a p-value can be calculated by comparing the experimental data to this frequency distribution. This statistical test serves as a simple model to directly analyze the influence of cell proximity on the prevalence and kinetics of IIF.

3.3 RESULTS

Comparison of IIF Behavior in Cell Suspensions and Confluent Monolayers

The cumulative incidence of IIF for V-79W cell suspensions and confluent monolayers at a constant subzero temperature is presented in figure 3.2. The kinetic response of these two cells at three experimental temperatures is shown in figure 3.3. Cells in suspension exhibited a lower incidence of intracellular ice formation at each time interval at constant temperatures when compared to cells in a confluent monolayer.

Influence of Cell-cell Contact on the Statistical Test

The mean normalized number of connected components is plotted as a function of temperature for the experimental and randomly calculated data (figure 3.4). The experimentally determined normalized number of connected components is significantly greater than the random number ($p<0.001$). At -8°C each cell that develops intracellular ice has more than 2 adjacent cells that also freeze intracellularly. This is compared to the random data where each frozen cell will have only 1.5 adjacent cells that form intracellular ice. With decreasing temperature, the normalized number of connected components increases as predicted by an increase in the cumulative incidence of IIF (figure 3.2).

Figure 3.5 presents the mean normalized number of connected components as a function of time for the experimental and randomly calculated data at three experimental temperatures. The experimentally determined number of connected components is significantly greater than the random number for all temperatures and at all times ($p<0.001$). As the temperature decreases, the normalized number of connected components at any one time interval increases, commensurate with an increase in the cumulative incidence of IIF (figure 3.3).

3.4 DISCUSSION

The qualitative observation that cell-cell contact facilitates the nucleation of intracellular ice in adjacent cells was investigated using a simple statistical method to test the degree of randomness of IIF in an *in vitro* tissue model. These results indicate that the distribution of cells displaying IIF occurs in a non-random fashion, with cells that develop intracellular ice being localized to clusters in the monolayer. Further, there is a greater proportion of frozen cells with adjacent cells that freeze at any given time then can be randomly attributed. Therefore, when one cell freezes, there is an increased likelihood that an adjacent cell will freeze. This implies that an induction mechanism which allows for the nucleation of adjacent cells by an already frozen cell can facilitate the increased incidence of IIF in monolayers.

There is evidence to support such a mechanism of ice induction between adjacent cells in tissues. Berger and Uhrik concluded that the spread of intracellular ice between neighbouring cells occurs as a result of the growth of ice through intercellular channels (16). It is proposed that gap junctions provide the means by which ice crystals can pass through membranes to nucleate adjacent cells (16) This supports the observation that the presence of cell

junctions plays an important role in the recovery of tissues (4). Since, the cell line used in this study does not form intercellular junctions, the induction of ice between neighbouring cells must also occur in the absence of channels between adjacent cells.

The formation of intracellular ice may result in damage to the cell membrane and that of adjoining cells allowing for the nucleation of ice through these damage sites. However, it has been previously noted that a large proportion of cells in a fibroblast monolayer can maintain their membrane integrity in the presence intracellular ice. The development of intercellular junctions would have allowed for the leakage of fluorescent dyes between adjoining cells and the dissociation between IIF and a loss in membrane integrity would not have been observed. In plant systems, it has been further shown that cells can undergo repeated freezing due to the lack of permanent damage done to the membrane resulting from IIF (25). It is unlikely then that 'artificial' junctions are formed between cells as a result of intracellular ice formation.

Toner *et al.* proposed that intracellular ice formation in cellular systems results from surface-catalyzed nucleation (11). In this model, the interaction of external ice with the plasma membrane is sufficient to nucleate internal ice without compromising the barrier properties of the cell membrane. This model, however, does not preclude the possibility that intracellular ice can function in a similar fashion. The internal ice may alter the structure of the membrane allowing for the external surface of the membrane to become an efficient ice nucleating site. The induction of ice between adjacent tissue cells might therefore occur as a result of the ice in one cell, using the adhesion between cells, to nucleate ice in adjacent cells.

The observation that cells in contact can effectively induce the formation of ice in adjoining cells without a disruption in the plasma membrane integrity supports the surface-catalyzed nucleation theory. The question then is how does the first cell in a cluster of cells freeze? In 1974, Brown and Reuter suggested that there were two types of cellular nucleation in tissues (18). The first one was the random nucleation of a cell without the influence from surrounding cells. The second, more predominate type, manifested itself as the sequential freezing of adjacent cells. The mechanism by which this initial random cell freezes is most likely similar to the mechanisms by which IIF occurs in single cells. The proposed theories include: ice growth from the extracellular environment into the cells via membraneous pores (26); the membrane acting as a nucleator when acted upon by extracellular ice (11), or the rupture of the membrane due to osmotic movement of water (10,27). Once a cell is nucleated, it can then induce ice formation in adjacent cells.

The formation of intracellular ice formation in tissues is a result of multiple mechanisms. The initial freezing of a cell occurs by the interaction of external ice with the cell membrane, but once a single cell is frozen, then neighbouring cells freeze as a result of the induction of intracellular ice. The enhanced nucleation of ice amongst adjoining cells will significantly increase the incidence of intracellular ice formation in tissues. Therefore, cell-cell contact plays a significant role in the reduced viability of cryopreserved tissues.

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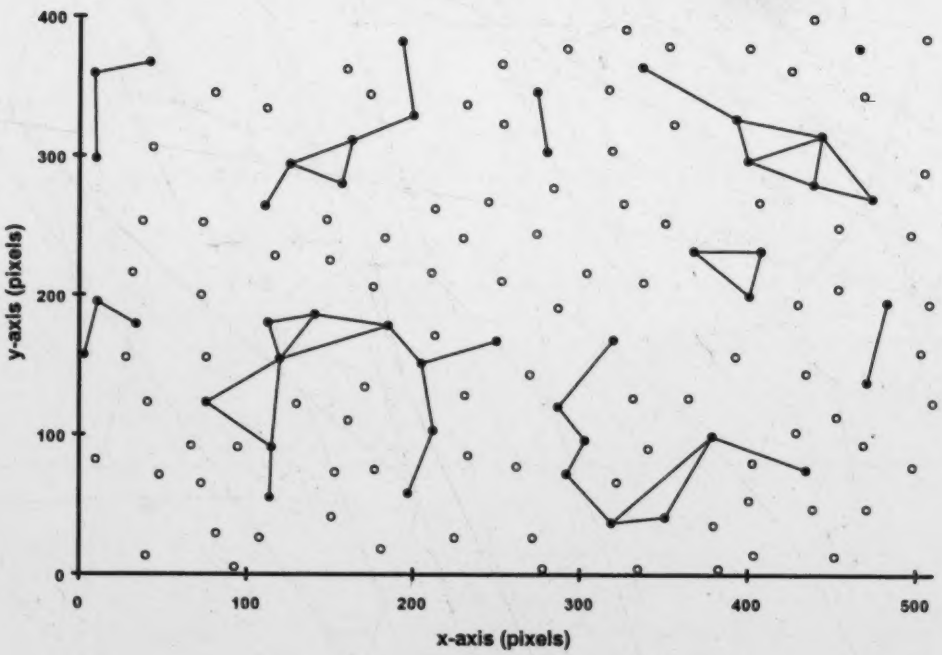

Figure 3.1 Summary of statistical analysis. The center of each cell in the fibroblast monolayers is plotted on a graph (open circle) and correlated with those cells that display intracellular ice formation (closed circle). Directly adjacent cells which display intracellular freezing are connected on the graph by a line. This data represents a V-79W fibroblast monolayer cooled at -25°C/min to -6°C and nucleated.

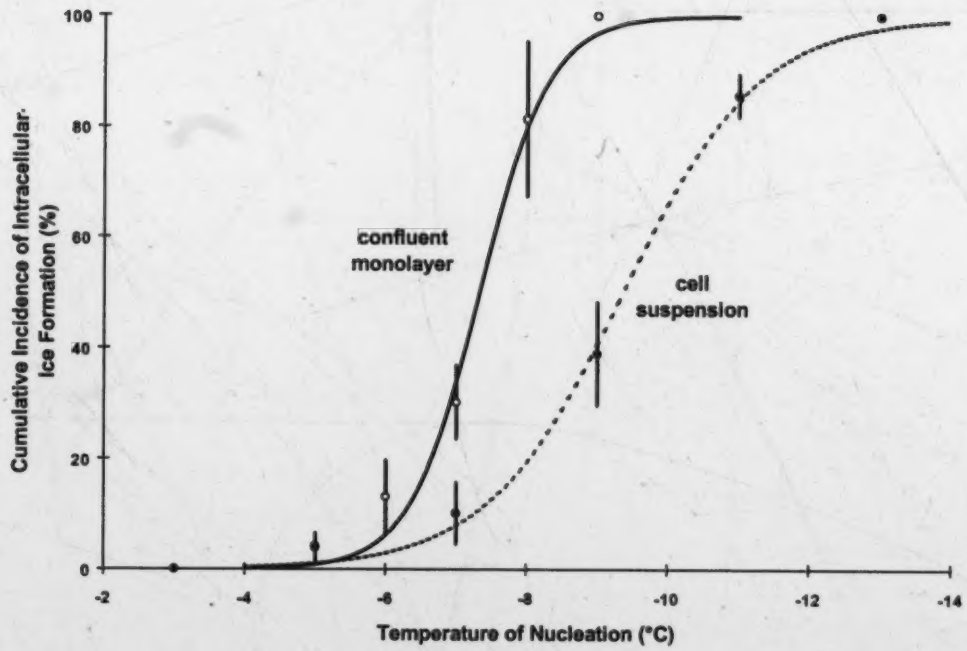

Figure 3.2 Cumulative incidence of intracellular ice formation in V-79W fibroblasts. The data points represent the cumulative incidence of IIF as a function of the experimental temperature, and the bars represent the standard error of triplicate samples. Lines are logistic curves fit to the experimental data.

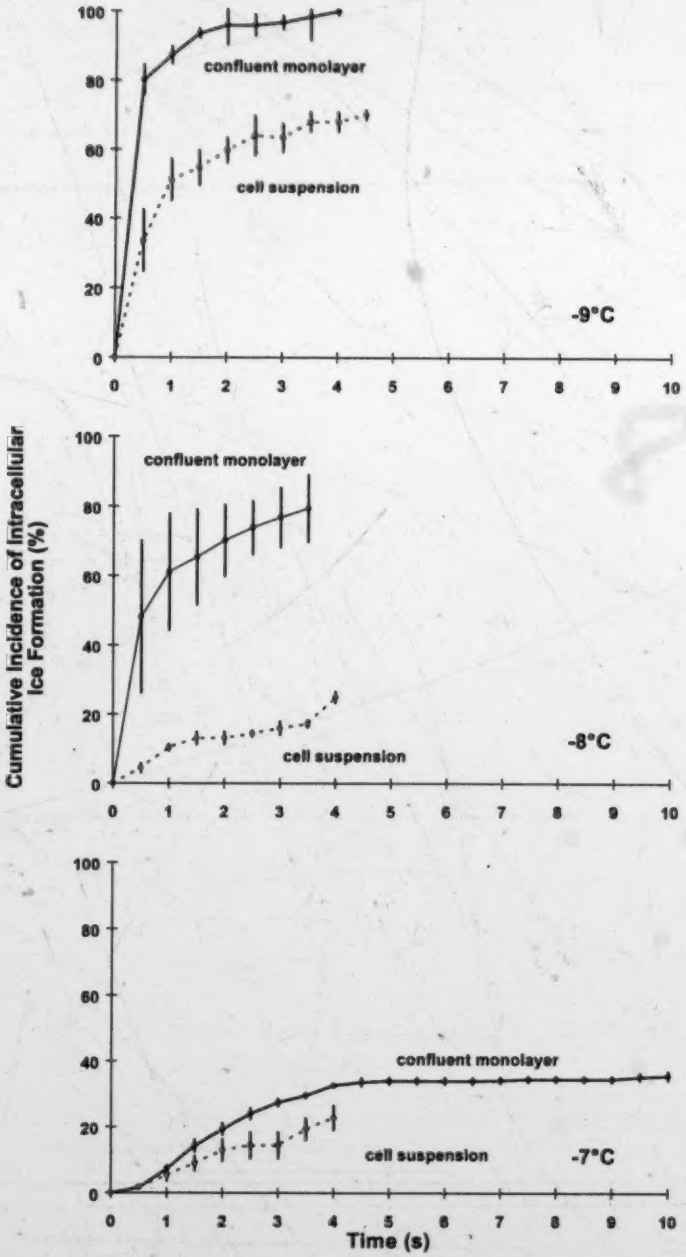

Figure 3.3 Cumulative incidence of intracellular ice formation as a function of time for V-79W fibroblasts. The cumulative incidence of IIF in cell suspensions and confluent monolayers as a function of time for three experimental temperatures (-7°C, -8°C and -9°C) is shown. The bars represent the standard error of triplicate samples.

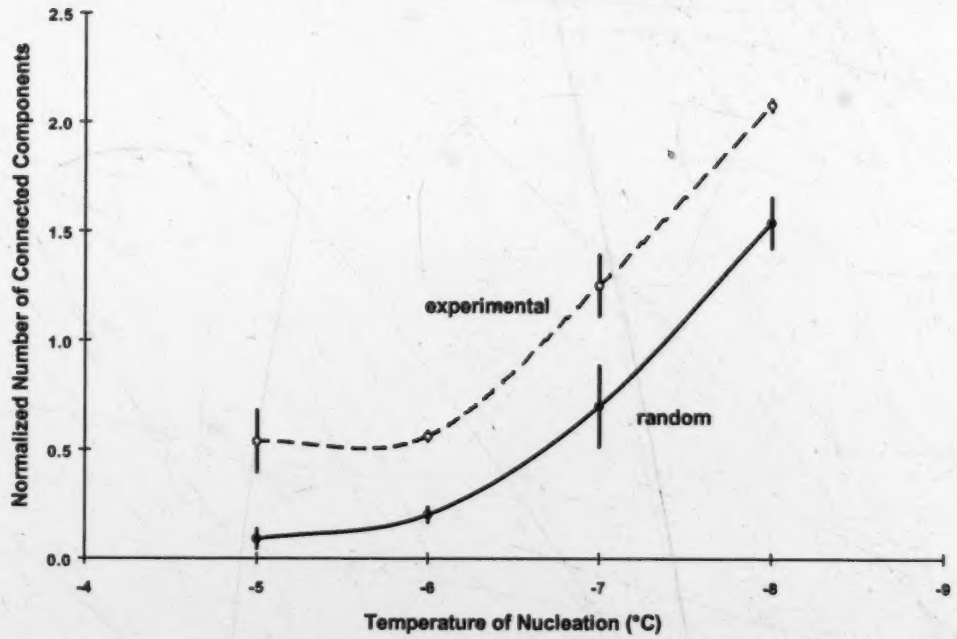

Figure 3.4 Normalized number of connected components as a function of temperature. The data points represent the mean normalized number of connected components, and the bars denote the standard error of triplicate samples.

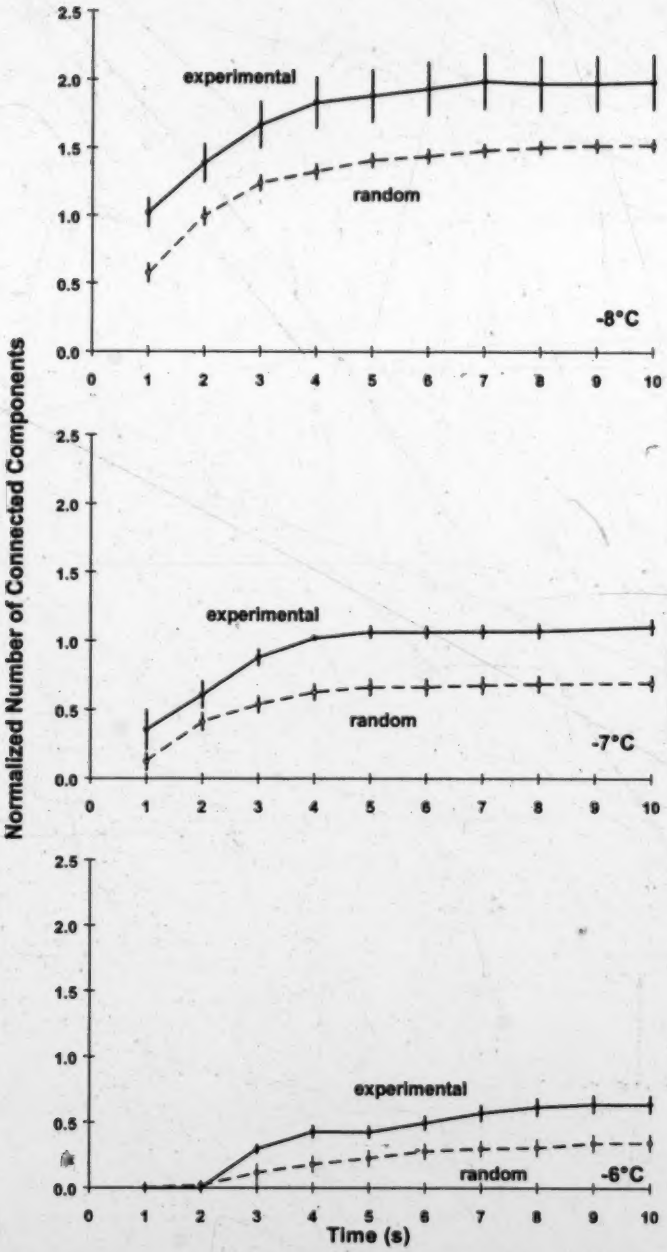

Figure 3.5 Normalized number of connected components as a function of time for three experimental temperatures. The data points represent the mean normalized number of connected components, and the bars denote the standard error of triplicate samples.

CHAPTER 4
INTRACELLULAR NUCLEATION THROUGH GAP JUNCTIONS

4.1 INTRODUCTION

The traditional approach to the cryopreservation of cells, tissues, and organs has been to use high concentrations of cryoprotectant and rapid cooling rates to reduce or eliminate intracellular ice formation. By balancing the toxicity of the cryoprotectants and osmotic stresses imposed during its addition and removal, a diverse number of cell types have been successfully cryopreserved. The application of this technique to tissues, however, has met with limited success. The ability to isolate and preserve cells from tissues, while being unable to preserve these same cells *in situ*, has begun to focus attention on the unique morphological and physiological characteristics of tissues.

A tissues is a complex, organized system of cells and structures. In connective tissues, sparsely distributed cells are arranged in an elaborate extracellular matrix which together facilitates the maintenance of the tissues structure and function. Cartilage, as an example, is composed of cells (chondrocytes), which function to sustain the composition of an extracellular matrix (1). The cartilage matrix is composed of collagen and proteoglycans which confers the mechanical properties of the tissue (1,2). In contrast, cells in epithelial tissues are bound by direct cell-cell adhesions, with only a thin extracellular matrix. The endothelial layer of the cornea is a continuous monolayer that maintains the degree of hydration of the corneal stroma (3). The distribution, type and function of cell junctions are therefore dependent on the type of tissue.

Cell-cell and cell-matrix adhesions are critical in a number of basic biological processes. Cell secretory activity, wound healing, morphogenic movement, cellular migration and cell-cell communication are all made possible by interactions between cells (4). Cell junctions can be classified into three functional groups: occluding junctions, anchoring junctions and communication junctions (4). Tight junctions (zonula occlundens) control the diffusion of molecules across the epithelium by acting as a barrier restricting the passage of molecules between cells, and by modulating the distribution of apical and basolateral membrane-bound carrier proteins. Anchoring junctions (zonula adherens, desmosomes and hemidesmosomes) connect the cytoskeleton of a cell to those of its neighbours or to the extracellular matrix. Finally, gap junctions allow cell-cell communication by permitting small molecules to pass directly from cell to cell. Cell junctions play a critical role in the physiology of tissues.

The presence of cell junctions in multicellular systems has been proposed as a contributing factor to their enhanced sensitivity to freezing injury. The mechanism by which damage occurs to tissues as a result of these cell-cell and cell-matrix adhesions has been the subject of debate. Disruption of the cell junctions by the physical and chemical changes that occur during freezing affects the viability of the tissue. Formation of extracellular ice in tissues and organs has been shown to disrupt the structure of tissues and severely inhibit survival (5). The intercellular junctions have also been shown to be affected by exposure to the cryoprotectants (6) and osmotic stresses (7,8) encountered during freezing. In contrast, the cell junctions themselves might be the cause of increased susceptibility to cryoinjury in tissues. Cell-cell and cell-surface interactions modulate the incidence of intracellular ice formation in tissue systems (9). Cell-

cell adhesions facilitate the nucleation of ice between adjacent cells, and it has been reported that the nucleation of ice in one cell, after a brief delay, is followed by ice nucieation in an adjacent cell (9,10,11,12,13,14).

In 1965 Mazur proposed that intracellular ice formation occurs as external ice passes through pores in the membrane (15). This model however, does not preclude the possibility that intracellular ice can function in a similar fashion. The propagation of ice between adjoining cells in tissues could occur via the movement of internal ice through gap junctions. The spread of intracellular ice from one cell to another in a sequential manner via intercellular junctions has been reported (11). Using single strands of salivary tissue, Berger and Uhrik demonstrated a regular induction of ice between cells and that this induction could be inhibited by the addition of agents that uncoupled adjacent cells (11). Gap junctions provide a means by which ice crystals can pass through membrane pores to nucleate adjacent cells.

The objective of this study is to investigate the intracellular nucleation through gap junctions in a two-dimensional model tissue system. The use of single strands of tissue, prevented Berger and Uhrik from further investigating the two and three-dimensional effects of cell adhesion on the induction of intracellular ice. This information will provide a clearer understanding of the mechanism(s) responsible for the induction of ice between neighbouring cells, and will facilitate the development of more accurate models that will allow for the design and implementation of techniques for the cryopreservation of tissues.

4.2 MATERIALS AND METHODS

Cell Culture

Two cell lines were used to investigate the role of cell-cell contact on intracellular ice formation. The first cell line is the V-79W line of Chinese hamster fibroblasts. These cells do not form occluding or cell-cell communication junctions (16). The culture technique for these cells is described elsewhere (chapter 2). Briefly, hamster fibroblast cells were maintained in culture, seeded at $6.7x10^3$ cells/ml and incubated for 5 d at 37°C to form confluent monolayers on coverslips.

The second cell line is the Madin- Darby Canine Kidney (MDCK; CCL 34) cells obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured at 37°C in an atmosphere of 95% air + 5% carbon dioxide in an antibiotic-free minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY). Cells were kept in tissue culture flasks (25 cm^2, Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C. For single cells attached to glass and cells in a confluent monolayer, sterilized cover slips (12 mm circle, FISHER Brand) were placed in a petri dish (100x15 mm, FISHER Brand) and covered with 15 ml of supplemented MEM containing $1x10^6$ cells. The petri dishes were kept in an incubator for 12 h to allow the cells to attach and 5 d to allow the growth of a monolayer. The MDCK epithelial cell has been previously shown to form occluding, anchoring and communication junctions under similar culture conditions (6,8)

Detection of Intracellular Ice Formation

The formation of intracellular ice results in the scattering of light (17,18). The traditional method for the detection of IIF has been the occurrence of a sudden darkening of the cytoplasm (17,18). However, when monolayers are assayed for the formation of intracellular ice, particularly at low subzero temperatures, the high proportion of cells displaying this 'flashing' makes it difficult to determine the IIF status of each cell in the monolayer. The use of a fluorescent nuclear stain, SYTO (Molecular Probes, Eugene, OR) and a CCD video camera (ZVS-47DEC, Carl Zeiss, Germany) is an effective method for the detection of intracellular ice formation (chapter 2). This technique was used in this study to quantify intracellular nucleation in monolayers of MDCK and V-79W cells.

Freezing at Constant Subzero Temperatures

The cryomicroscope used for this study is described in detail elsewhere (chapter 2). Briefly, it consisted of a Zeiss light microscope (Carl Zeiss, Germany), a low-light CCD video camera, a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar in design to that described by Diller (19,20). The MDCK and V-79W cell monolayers were stained with 1.25 μM SYTO stain and allowed to incubate at 37°C for 15 min and 2 min respectively. This was previously determined to be sufficient time to allow for the diffusion of the stain. The coverslips containing the cultured single attached cells or cell monolayers were then placed inverted on the cryostage. Similarly, a 7 μl sample of the MDCK or V-79W cell suspension was added to the cryostage and protected with a glass coverslip. The cryostage was cooled at -25°C/min to a predetermined subzero temperature where it was held for 10 s. The sample was then nucleated at the constant temperature using a cold copper probe and the formation of

intracellular ice was observed. The entire procedure was videotaped for later analysis and both brightfield and fluorescent images were recorded of the unfrozen and frozen samples. Still images were captured for analysis from videotape using a TARGA+ image capture system (Truevision, Indianapolis, IN).

The proportion of cells which froze intracellularly and the velocity of the ice front were determined experimentally for each temperature. To measure the ice interface velocity, distance on the television was calibrated with distance on the microscope using a micrometre. The distance over which the ice traveled in one video frame (1/30 s) was measured and the rate was determined.

4.3 RESULTS

Intracellular Ice Formation at Constant Temperature

The cumulative incidence of intracellular ice formation as a function of temperature for the V-79W fibroblast and MDCK epithelial models investigated is shown in figure 4.1. The fibroblast data confirms the reports that the physical contact of one cell with another affects the proportion of cells exhibiting intracellular ice formation (9). For the MDCK cell line, there was an increase in the number of single attached cells displaying IIF versus those cells in suspension. Of interest, is the presentation of IIF in the MDCK monolayers. At subzero temperatures as high as -4°C, 100% of the cells in the monolayer formed intracellular ice.

The MDCK monolayer exhibited a significantly greater proportion of cells displaying IIF at high subzero temperatures (-4°C to -8°C) than the V-79W monolayers ($p<0.001$ - student's t test).

Ice Propagation in Monolayers

The pattern of ice propagation through the MDCK monolayers was significantly different than that in the fibroblast monolayers. In the V-79W monolayer, the passage of the ice front through the field of view was followed by nucleating events in a number of randomly dispersed cells. This initial formation of ice was proceeded by the induction of ice from one cell to another resulting in clusters of cells displaying IIF. However, in the MDCK monolayer, the passage of the ice front was quickly followed by the formation of ice in the monolayer in a wave-like manner in the same direction.

The approximate rate of the passage of the ice front, and the formation of intracellular ice as a function of temperature are shown in Table 4.1. The rate of passage of the ice front is dependent on the temperature, with the faster rate occurring at a lower temperature (21,22). The rate of propagation of intracellular ice, however, is independent of temperature.

Discussion

The presence of gap junctions in cell monolayers facilitates the induction of intracellular ice between adjacent cells. The MDCK monolayer demonstrated a significantly greater proportion of cells displaying IIF at higher subzero temperatures than was shown in the two other cell models which lacked cell-cell contact. The cumulative incidence of IIF in the MDCK monolayer was also substantially higher than that in the V-79W monolayer. The two cell lines demonstrated a significantly different degree of dependency on temperature for the development of intracellular ice. This suggests that the mechanism by which intracellular ice forms in the MDCK epithelial cells occurs more readily than that in the V-79W fibroblasts cells. Further, the pattern of intracellular nucleation was

also effected by the presence of gap junctions in the MDCK monolayer. In the MDCK monolayer, the formation of intracellular ice followed a definite pattern and occurred at a constant rate independent of temperature. This induction process was more ordered compared to the V-79W monolayer, where a random initial dispersion of cells displaying IIF took a significantly longer time to induce nucleation in adjoining cells (9). The comparatively short delay (10-20 ms) and the temperature independent nature of the induction of ice between adjoining cells in the MDCK monolayer indicates that gap junctions play a significant role in the induction of ice between adjoining cells.

The mechanism by which gap junctions facilitate intracellular nucleation in adjoining cells has been recently suggested by Berger and Uhrik (11). They concluded the 200-300 ms delay in the propagation of ice between adjacent salivary cells was indicative of the ability of ice to transverse the cell membranes via gap junctions in a manner similar to Mazur's pore theory of IIF nucleation (15). The rate, pattern and cumulative incidence of formation of intracellular ice in the MDCK monolayer supports this mechanism for the induction of ice via gap junctions. However, the temperature-independent nature of the observed induction of intracellular ice in the MDCK monolayer, would suggest that the angle of contact of the intracellular ice and the gap junctions is more dominant than the cooling rate dependent reduction in the ice crystal size. Intuitively, the formation of intracellular ice in the two-dimensional monolayers would restrict the directional plane of growth of ice and could result in an angle of contact between the intracellular ice and the gap junctions approaching 90°. This would permit the relatively uninhibited propagation of ice between adjacent cells and result in the

observed cumulative incidence of intracellular ice formation seen in the MDCK monolayers.

Tissues that form gap junctions are particularly at risk to the relatively small degree of supercooling that occurs at high subzero temperatures. The probability of nucleation of an isolated cell exposed to 3 degrees of supercooling is relatively low for most cell types. For the MDCK cell line, none of the cells in suspension form intracellular ice at -3°C, but, approximately 100% of the cells in a monolayer form intracellular ice when exposed to the same temperature. The ability for gap junctions to facilitate the induction of ice between adjoining cells allows for this enhanced degree of ice nucleation in monolayers. The formation of ice in a single cell is all that is required to initiate this process. This phenomenon has practical applications when applied to traditional cryopreservation protocols which involve high concentrations of cryoprotectant and rapid cooling. In studying the effects of cooling rates on the survival of keratocytes from rabbit corneas, Armitage observed that the cooling rate for maximal survival of cells in a monolayer was significantly less than that for cells in suspension (23). The freezing of tissues will require special conditions to reduce the degree of supercooling and the subsequent nucleation and propagation of ice from a single cell to the entire tissue.

The ability of ice to propagate between cells via intercellular junctions provides further evidence that there exists a multiple number of mechanisms by which intracellular ice can form in tissues. It has been suggested that the mechanism by which the first cell in a monolayer freezes is similar to the proposed mechanisms for IIF in single cells (11). This can occur as a result of ice entry into the cell via pores (15), membrane failure (22,24,25), or surface-catalyzed

nucleation (26). However once this initial random cell has frozen, the induction of ice from one cell to another can result. In tissues that lack cell-cell communication junctions, surface-catalyzed nucleation can facilitate the initiation of ice in adjacent cells (chapter 3). In tissues that are coupled as a result of gap junctions, cell-cell nucleation via these connections can readily occur. Damage to tissues as a result of intracellular ice formation is likely a culmination of these multiple mechanisms.

Although questions remain as to the means by which IIF forms and the mechanism by which it damages cells (27), a common observation in the freezing of a wide variety of cells and tissues is that intracellular ice is lethal. By facilitating the nucleation of ice amongst adjoining cells, intercellular junctions can enhance the incidence of intracellular ice in tissues. This induction of ice between neighboring cells *in situ* would result in massive damage to the cells and a reduction in tissue viability after freezing. While isolated kidney cells (28), cardiomyocytes (29,30), corneal keratinocytes (23) and hepatocytes (31,32) can recover partial viability after freezing, an inability to preserve these cells *in situ* (33,34,35) is likely the result of gap junction enhanced induction of IIF between adjacent cells. Improvement of the viability of these and other tissues will require a more detailed examination of the induction process and the mechanism of damage cause by intracellular ice formation.

Tissues are a complex arrangement of multiple cell types whose interaction together dictates their overall function. During freezing, these cell-cell and cell-matrix adhesions affected the incidence of intracellular ice formation resulting in tissue damage and lethal injury to the constituent cells. An ability to cryopreserve tissues will depend on the elucidation of the mechanisms by which the chemical

and physical changes manifested during freezing affect the biological and biochemical components of tissues. The cryophysiology of tissues must be carefully studied if successful cryopreservation of these complex systems is to be realized.

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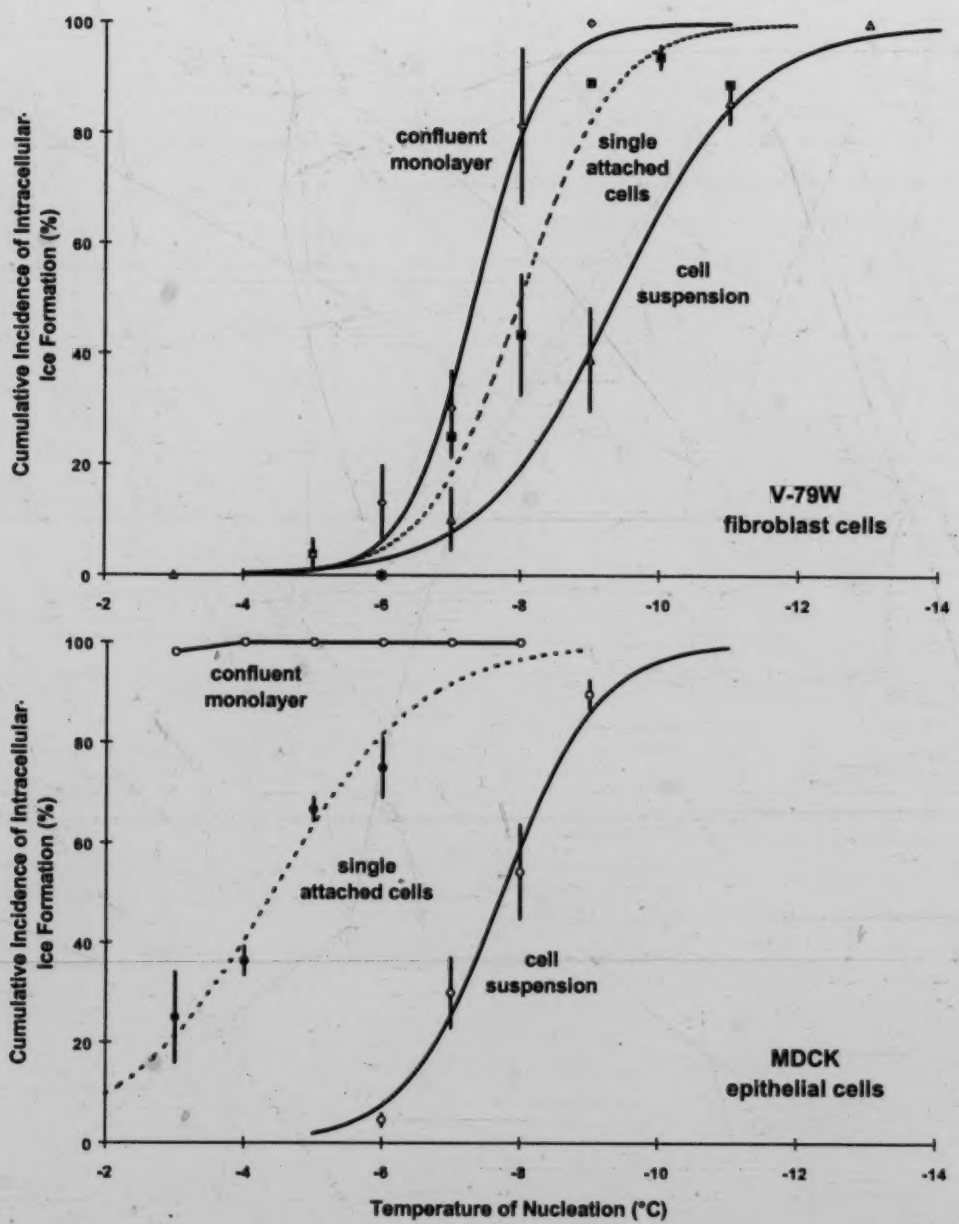

Figure 4.1 Cumulative incidence of intracellular ice formation in V-79W fibroblasts and MDCK epithelial cells. The data points represent the cumulative incidence of IIF as a function of the experimental temperature, and the bars represent the standard error of triplicate samples. Lines are logistic curves fit to the experimental data.

Temperature (°C)	Rate of Ice Growth ($x10^{-3}$ m/s)	Rate of Intercellular Nucleation ($x10^{-3}$ m/s)
-5	2.80 ± 0.45	1.50 ± 0.15
-6	3.60 ± 0.30	1.20 ± 0.30
-7	4.80 ± 0.15	1.50 ± 0.15

Table 4.1 The table compares the rate of growth of the ice front and the rate of intercellular nucleation in the MDCK monolayer as a function of temperature. The standard error of triplicate samples is denoted.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

5.1 REVIEW OF THESIS OBJECTIVES

The objective of this thesis was to explore the role that cell adhesions have on the mechanism of intracellular ice formation in tissue systems. The hypothesis stated that the induction of intracellular ice formation between adjacent cells via cell-cell interactions is responsible for the enhanced sensitivity of tissues to freezing. Investigations were conducted to explore the role that cell adhesions had on modulating the integrity of the plasma membrane and the initiation of intracellular ice. The pattern and distribution of intracellular ice formation was analyzed to determine the role of cell-cell contact and communication junctions. From this work, the influence of cell adhesions on the formation of intracellular ice and the mechanisms by which this occurs in tissues was investigated.

5.2 SUMMARY OF RESULTS

Cell junctions have a distinct role in a tissues ability to respond to low temperatures. The development of cell-cell and cell-matrix interactions significantly enhances the incidence of intracellular ice formation in tissues. The damage associated with this ice formation is a contributory factor to a tissue's heighten sensitivity to freezing. This thesis explored the effects of cell junctions on the mechanisms of intracellular ice formation in tissues.

Current theories on the mechanism of intracellular ice formation in isolated cells involve the interaction of external ice with the plasma membrane. The unique morphology of *in situ* cells was investigated to determine the susceptibility of cells to membrane damage and the subsequent formation of intracellular ice. The formation of intracellular ice in cell suspensions and single attached cells

corresponded directly with a loss of the membrane integrity. However, the formation of intracellular ice in tissues did not immediately cause or result in damage to the plasma membrane of the constituent cells. This identified the need for further investigation of the mechanism by which intracellular ice forms in tissue systems, and the role that cell junctions have in this process.

To investigate the effects of cell junctions on the incidence of intracellular ice formation it was necessary to devise a technique for the detection of IIF in tissue systems. The current 'flashing' methods for assaying IIF was inadequate due to the close packing of cells. The use of a fluorescent nuclear stain, SYTO and a CCD video camera was shown to be an effective alternative method for the detection of intracellular ice formation in monolayers.

The influence of cell-cell contact on the incidence of intracellular ice formation in confluent monolayers was investigated using a simple statistical method. It was found that as one cell freezes, there was an increased probability that an adjacent cell would freeze. This demonstrates that induction of ice between adjoining cells occurs and supports the enhanced incidence of intracellular ice observed in the *in vitro* tissue models. It was proposed that the induction of ice in monolayers without communication junctions occurs as a result of surface-catalyzed nucleation.

In tissues that develop communication junctions, the incidence of intracellular ice formation was significantly enhanced. In MDCK monolayers it was shown that gap junctions can facilitate the nucleation of ice in adjacent cells. This induction occurs much more readily than the surface-catalyzed nucleation seen in the V-79W monolayers, and highlights the increased sensitivity to injury that tissues with gap junctions encounter when exposed to low temperatures.

This study has shown that the intracellular nucleation of cells *in situ* can occur by a multiple number of mechanisms. The induction of ice between adjacent cells occurs after the interaction of external ice with the membrane which results in the formation of ice in a single cell. Surface-catalyzed nucleation or induction via gap junctions, promotes the induction process. Cell junctions facilitate this induction of intracellular ice to adjoining cells and therefore are responsible for the increased incidence of intracellular ice formation observed in tissues.

5.3 SIGNIFICANCE TO CRYOBIOLOGY

Current attempts at tissue cryopreservation are modeled on the assumption that there is a direct correlation between the biophysical and physiological responses of cells and tissues to freezing. This thesis has demonstrated that this is not the case. A tissues is a complex, organized system of cells and structures. The cell-cell and cell-matrix interactions, which are critical to the basic biological processes of the tissue, are also crucial to the successful management of the physical and chemical changes that occur during freezing. These cell adhesions have been demonstrated to be important determinants in the formation of intracellular ice in tissues and hence influence the ability of tissues to respond successfully to freezing.

This thesis has provided a clearer understanding of the mechanisms responsible for the induction of ice between adjacent cells in a tissue. With this information, more accurate mathematical models can now be developed which may be used to test ideas and assist in the advancement of techniques for the cryopreservation of tissues. But most importantly, this work has affixed the concept of cryophysiology to the vocabulary used in cryobiology. The intricate elements that compose cells and tissues - the physiological components - are

fundamentally important in the successful application of low temperatures to these systems. We can no longer be solely concerned with the physical changes that occur during freezing, but must develop our understanding of the biological impact of these changes.

This work has established the necessity for the more complete understanding of the biochemical and physiological constituents of tissues. As the study of heat and mass transfer characterized the field of cryobiology for the past fifty years, cryophysiology will establish itself as the central element in the next phase of low temperature research. Advancements in this area will open the possibility for detailed investigations of the sites of damage in tissues during osmotic and thermal stresses encountered during cryopreservation. It will allow for the promotion of strategies for intervention to prevent or repair damaged sites, and will define the selection criteria for appropriate conditions for optimal cryopreservation. The ideas presented herein will hopefully stimulate not only the search for improved techniques for the cryopreservation of tissues, but more importantly, a desire to clearly understand the response of biological systems to low temperatures.